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TITLE OF THESIS SYNTHESIS AND STUDIES OF MODELS FOR TWO ZINC-CONTAINING METALLO-ENZYMES: ALCOHOL DEHYDROGENASE AND CARBONIC ANHYDRASE

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SYNTHESIS AND STUDIES OF MODELS FOR TWO ZINC-CONTAINING METALLO-ENZYMES: ALCOHOL DEHYDROGENASE AND CARBONIC ANHYDRASE

by

Ⓒ NEVILLE J. CURTIS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled SYNTHESIS AND STUDIES OF MODELS FOR TWO ZINC-CONTAINING METALLO-ENZYMES: ALCOHOL DEHYDROGENASE AND CARBONIC ANHYDRASE submitted by NEVILLE J. CURTIS in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry.

ABSTRACT

Several Zn^{++}-containing metallo-enzymes are known in which histidine imidazole nitrogens are ligands to the active site zinc. Two such enzymes are alcohol dehydrogenase (ADH), containing one histidine and two cysteine sulphurs, and carbonic anhydrase (CA), containing three histidines coordinated to Zn^{++}. Considerable effort has been applied to the synthesis and studies of small molecules which contain an imidazole (or pyridine), along with other ligating species as models which mimic the metal binding site and the enzyme process.

The first chapter deals with the synthesis and physical studies of chelating ligands containing a basic nitrogen and two sulphurs as models for ADH. This enzyme catalyses the interconversion of alcohols and aldehydes/ketones, using $NADH/NAD^+$. Whereas the reduction reaction has been successfully reproduced in model systems with nitrogen containing ligands (including a pendant carbonyl group), no model studies published to date have investigated the role of, or included, the sulphurs. Initially, ligands containing a pyridine and two thioether groups were synthesized but these were found not to bind Zn^{++} strongly enough for further study. Next, ligands containing pyridine or imidazole

and two thiols were made, however the Zn^{++} complexes of these precipitated from aq. solution even at low pH and no reduction, in aq. HMPA solution, of a carbonyl containing ligand of this form was found. Titrations of 1:1 complexes of these ligands (with Zn^{++}) showed that at low pH's both the thiol ligands were completely deprotonated. Since these observations cast doubt on the electrophilic role of zinc in enzyme catalysis (because of charge neutralization) a new mechanism of action was proposed in which one thiol is not bound to Zn^{++} in the reduction step. To counteract the effects of precipitation and charge neutralization, ligands containing a pyridine and only one thiol were prepared. However, again the required reduction could not be achieved and instead a complex reaction involving thiol and NADH analogue was seen.

The second chapter deals with a new protection group for imidazole nitrogen. The N (-diethoxy methyl) group is readily introduced and the corresponding 2-lithio imidazole anion was found to react with a variety of electrophilic agents to give good yields of 2-substituted imidazoles after deprotection. Deblocking was achieved via an acidic aqueous work-up, or, if necessary, could be effected under completely neutral conditions.

This method was used to make the acid-sensitive

tris(4,5-di*iso*propylimidazol-2-yl) phosphine which had been previously shown in a preliminary study to be an effective model for CA. The third chapter deals with a detailed study of the physical properties and catalytic activity (CO_2/HCO_3^- interconversion) of the Zn^{++} and Co^{++} complexes of this ligand. The catalysis was found to be first order in [Zn^{++} complex] and pH dependent, the bell shaped curve obtained by plotting k_{cat} versus pH indicating a deficiency in Zn^{++}-binding. The catalytic activity could be inhibited by halide ions in the order $Cl^- > Br^- > I^- > F^-$ and it was proposed that the inhibitor occupied a binding site on Zn^{++} to give an inactive species. The Co^{++} complex was also found to be active and inhibited by Cl^-. Although no experimental support for any one reaction mechanism was found, it was noted that the presence of a protein chain (as in the active site cavity) is not a prerequisite for activity, at least in this model.

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NOTES AND ABBREVIATIONS

Note : In all cases, for simplicity, divalent metal ion charges have been omitted in the diagrams

NADH : Nicotinamide-adenine-dinucleotide (reduced form)

NAD^+ : Nicotinamide-adenine-dinucleotide (oxidized form)

ADH : Alcohol dehydrogenase

LADH : Liver alcohol dehydrogenase

YADH : Yeast alcohol dehydrogenase

CA : Carbonic anhydrase

HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

MES : 2-(*N*-morpholino)ethanesulphonic acid.

LDH : Lactate dehydrogenase

BNAH : *N*-Benzylnicotinamide (reduced form)

PNAH : *N*-Propylnicotinamide (reduced form)

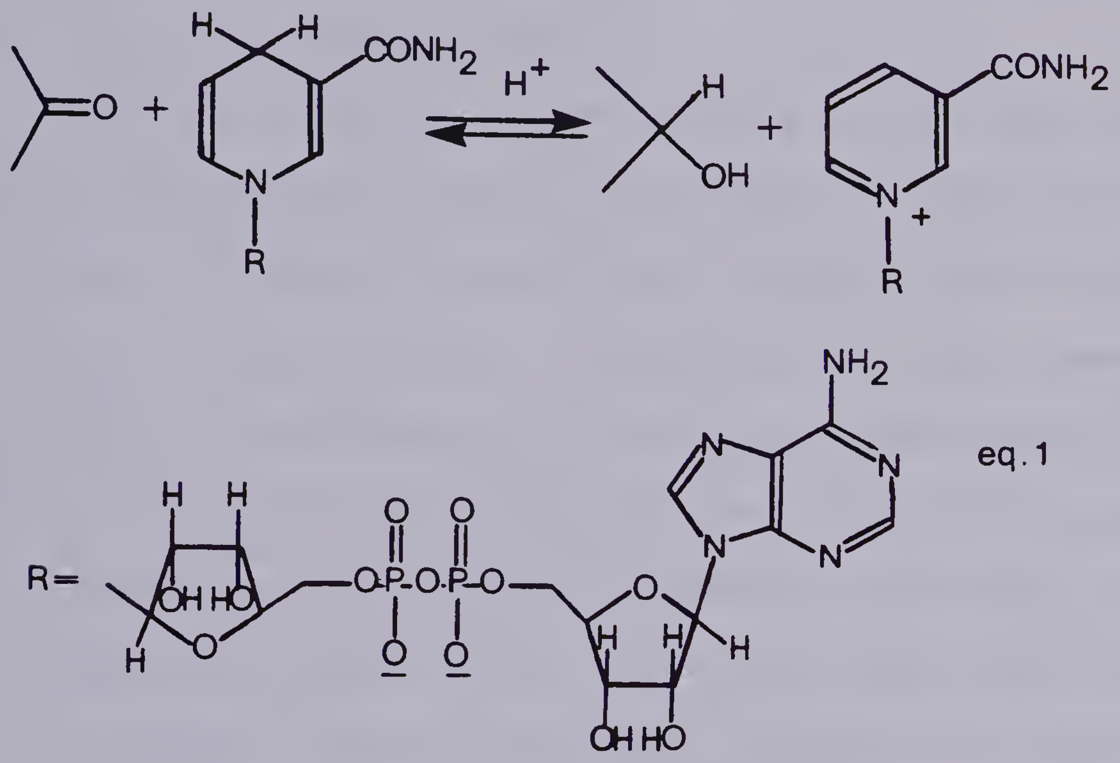
BCP : Bromocresol purple

SYNTHESIS AND STUDIES OF MODELS FOR ALCOHOL DEHYDROGENASE

INTRODUCTION

General

Alcohol dehydrogenases (ADH's), E.C.1.1.1.1., catalyse the interconversion of alcohols with the corresponding carbonyl compounds. The cofactors used are NADH and NAD$^+$ (eq. 1).



ADH's are widespread in nature and their properties have been reviewed extensively.[1] They have been found in bacteria, fungi, plants, insects, amphibians, mammals and birds. Of these, only those enzymes derived from yeast and mammalian liver sources have been investigated in detail.

The oxidation of alcohols by animal tissue was first

investigated in the late nineteenth century.[1a] Further work indicated the presence of an ethanol oxidizing enzyme in brewers yeast and in 1937 the first yeast alcohol dehydrogenase (YADH) was crystallized.[2]

Another source of the enzyme is the mammalian liver. The liver of a 70 kg human contains about 1 g of liver alcohol dehydrogenase (LADH) per kg weight and can metabolize about 7 g ethanol per hour.[3] The horse liver enzyme (HLADH) has been studied extensively since its crystallization in 1948.[4]

HLADH and YADH have some notable differences, the former being dimeric whilst the latter is made up of four sub-units. Substrate specificity between these enzymes and their isozymes varies a good deal but this seems to be due to differences in the size of the active site pocket rather than any major changes in chemical action.

The active site pocket in YADH is relatively small[5] and hence the enzyme is most effective with small substrates such as ethanol and acetaldehyde. LADH's are a lot less substrate selective, the active site pocket is larger and a wide range of alcohols and ketones (or aldehydes) may be interconverted. For example, an isozyme of HLADH is effective in the oxidation of 3-β-hydroxy steroids.

Both YADH and LADH show some stereoselectivity,[1a,d] eg., only one isomer of butan-2-ol is a substrate for YADH[6] (but both are substrates for LADH) and some large,

chiral ketones are enantioselectively reduced by LADH.[7] This has been utilized to effect conversion of 1-deuterioacetaldehyde to optically pure (1S)1-deuterioethanol in quantitative yield by YADH action.[8]

Chemical structure of the enzyme

One isozyme of HLADH has been fully sequenced.[9] It consists of two identical sub units, each containing 374 amino-acid residues. Several X-ray crystal structures have been determined and are consistent with this sequence.[1b,10,11] Partial sequences of the human and rat enzymes show homologies with HLADH of 90 and 80% respectively.[1b]

ADH's are metallo-enzymes, each sub-unit containing two zinc ions, both of which are essential for enzyme activity. The X-ray structures of the horse apoenzyme (no NADH/NAD$^+$) to 2.9 Å [10] and 2.4 Å [11] have been published and show the positions of the two zinc ions. One is coordinated by four cysteine ligands and a purely structural role has been proposed. The second zinc is at the active site and is found about 25 Å below the enzyme surface at the bottom of a mainly hydrophobic pocket. This zinc ion is coordinated in a distorted tetrahedral fashion by one histidine nitrogen and two cysteine sulphurs, the fourth site is said to be occupied by a water molecule.[10,11]

The X-ray analysis of a holoenzyme analogue[12] (enzyme/NADH/DMSO ternary complex) is most informative and shows that the substrate analogue DMSO replaces the water as a ligand to zinc. The NADH is situated nearby but is not a ligand to zinc (fig. 1). That this is indeed the active site is supported by X-ray evidence which shows that all enzyme complexes with coenzymes and/or inhibitors exhibit binding in this pocket. Chemical modification of the active site renders the enzyme inactive. Chelation of the zinc by 1,10-phenanthroline (giving a five coordinate zinc after expulsion of water[13]) destroys the activity, as does selective carboxymethylation of cys 46.[14,15]

The enzyme is deactivated by the loss of the active site zinc, the resultant protein being prone to air oxidation, presumably of cysteine residues. This zinc may be replaced by cobalt(II) and cadmium(II) to give catalytically active species.[16]

Although YADH has not been examined by X-ray methods, chemical analysis suggests a similar active site to that of LADH. Two cysteine thiols (per sub-unit) can be alkylated with loss of enzyme activity[17a] and the presence of a histidine group near or at the active site has been suggested.[17]

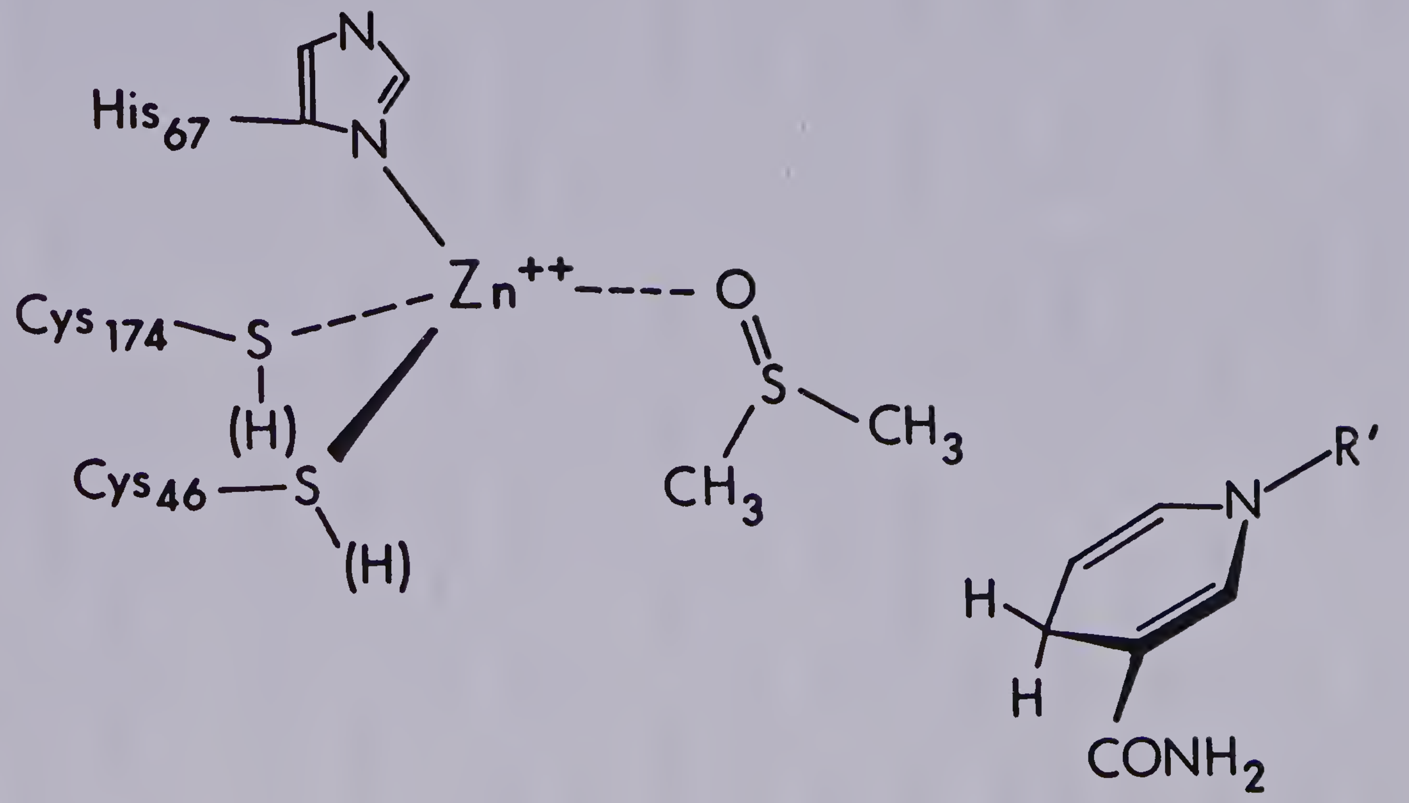

His67
N
N
Zn++
O
S
CH3
CH3
Cys174
S
(H)
Cys46
S
(H)
N
R′
H
H
CONH2

Fig. 1. The active site structure of a DMSO inhibited ternary complex of horse liver alcohol dehydrogenase depicting the Zn^{++} binding site (ref. 12).

Proposed mechanisms of action

NADH will not reduce normal ketones in the absence of the enzyme and the enzyme is not active without the metal ion. It was proposed[19] in 1957 that the zinc ion acts as a Lewis acid catalyst which polarizes the carbonyl bond to facilitate the transfer of hydride from NADH in the reduction reaction. A similar Lewis acid role for zinc has been proposed for other zinc metallo-enzymes such as δ-aminolaevulinate dehydratase, carbonic anhydrase, carboxypeptidase and alkaline phosphatase.[18d,e]

Kinetic analysis of the mechanism of the enzyme reaction is difficult in most cases, the rate limiting step being the dissociation of the coenzyme. However, some information has been gained using transient kinetics and a chemically modified version of the enzyme.[20] The equilibrium constant for the reaction has been measured by several workers. Racker[21] determined a value of 8 x 10^{10} M at pH 7.9 for ethanol oxidation with HLADH. Thus the reduction is favored at lower pH (6) and the oxidation at higher pH (8).

The enzyme is subject to conformational changes as a function of pH[22] and on binding coenzyme.[23] Binding of NAD^+ causes the reduction in pK_a of an ionizing group from 9.6 to 7.6[24,25] but this effect disappears for the metal free apoenzyme although the coenzyme is still

strongly bound in the absence of zinc.[18a] This behavior is to be expected: introduction of another positive charge in the hydrophobic environment should cause a reduction in pK_a.

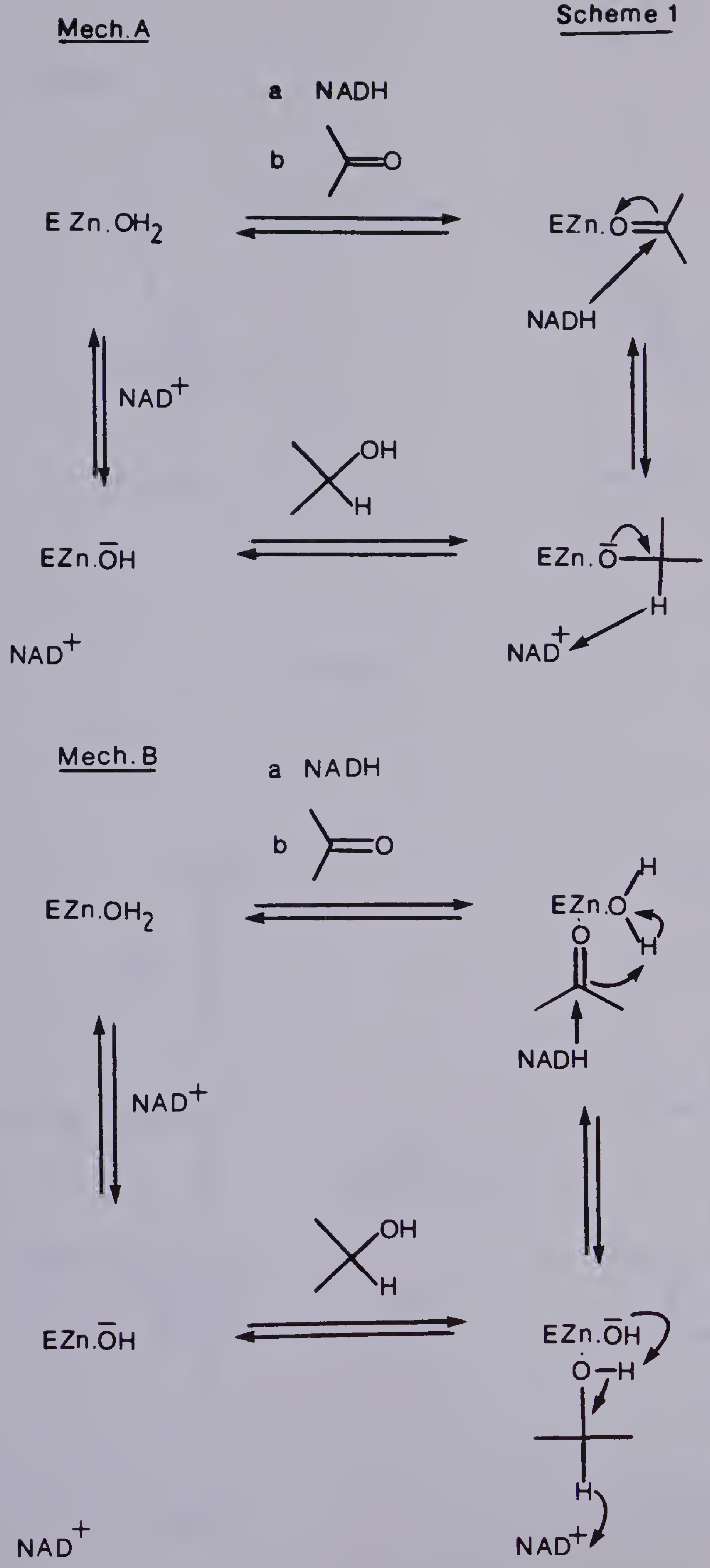
In view of the above data, three general mechanisms based on the electrophilic role of zinc have been proposed[18d] (Scheme 1). Only the redox step is formulated by most authors, however in order to show that the principle of microscopic reversibility is obeyed, a full cycle of steps is shown, assuming the following order for the reduction[1b,26]: 1. Enzyme binds NADH, accompanied by a conformational change. 2. Carbonyl binds. 3. Redox reaction occurs. 4. Product dissociates. 5. NAD^+ dissociates accompanied by a conformational change and the uptake of a proton by the enzyme.

In mechanism A, ketone displaces water as a ligand to zinc and then hydride transfer occurs giving the alcoholate species. Subsequently, hydrolysis liberates the alcohol.[18a]

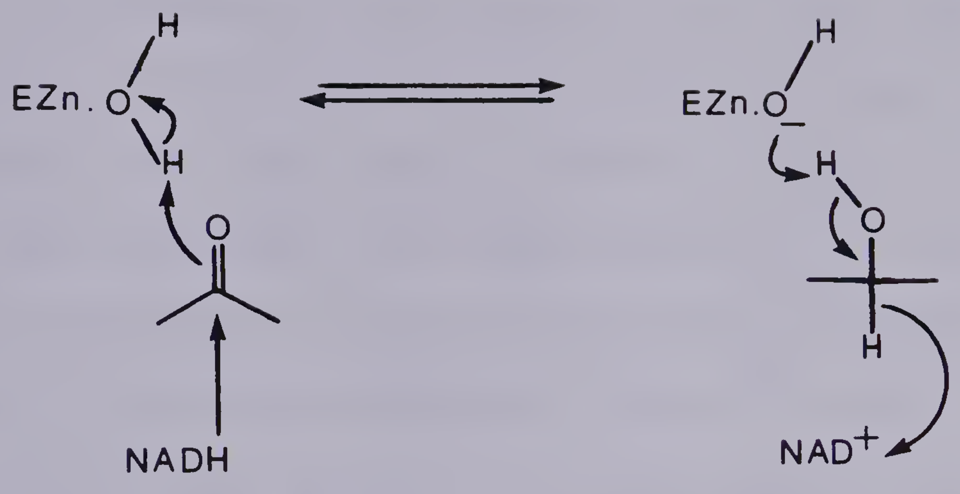
In mechanism B, the redox reaction takes place at five coordinate zinc, for which precedence occurs,[13] and the hydride transfer and protonation steps are concerted.[20,18d]

In mechanism C, the hydride transfer and protonation steps are again concerted but in this case the substrate is not directly bound to zinc.[18d,27]

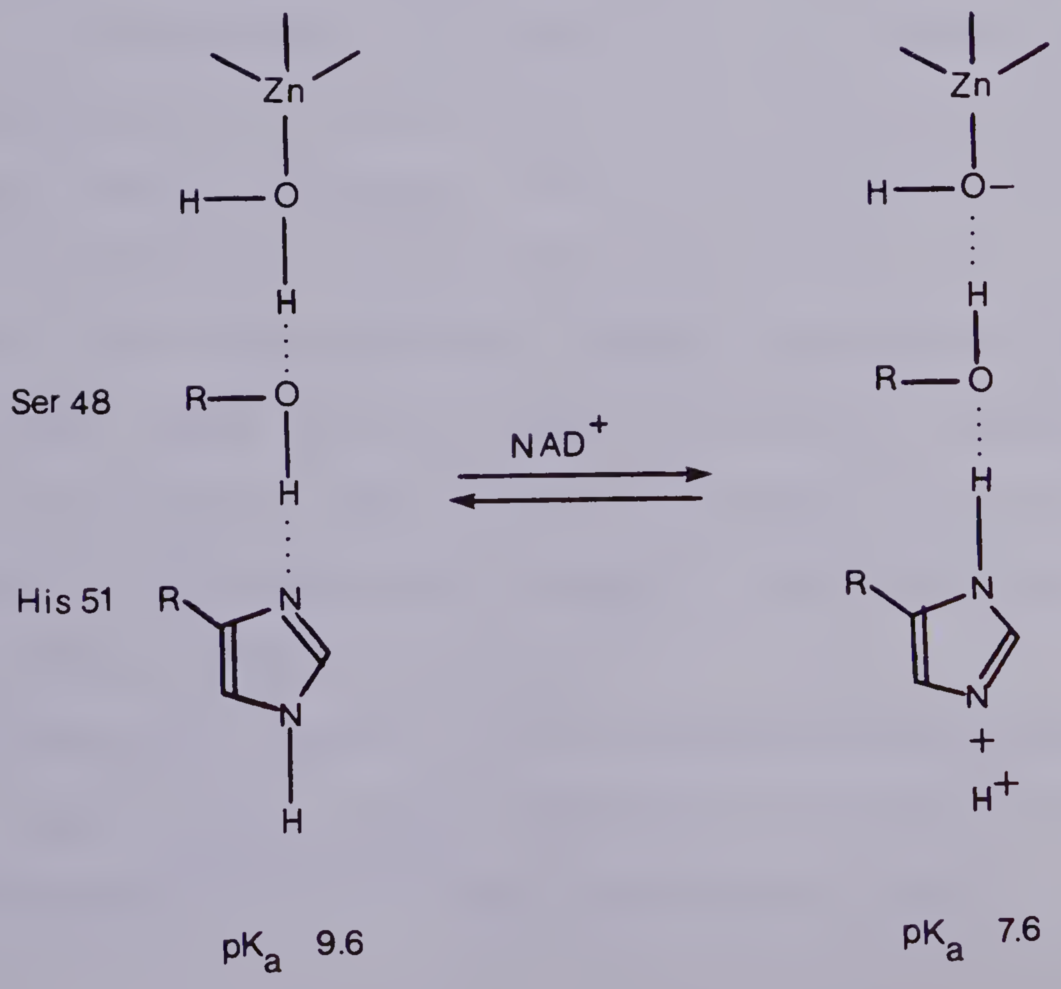
Scheme 1


Mech. A
a NADH
b
E Zn.OH$_2$
EZn.O
NADH
NAD$^+$
OH
H
EZn.$\bar{O}$H
EZn.$\bar{O}$
H
NAD$^+$
NAD$^+$
Mech. B
a NADH
b
EZn.OH$_2$
EZn.O
H
H
O
NADH
NAD$^+$
OH
H
EZn.$\bar{O}$H
EZn.$\bar{O}$H
O—H
H
NAD$^+$
NAD$^+$

Mech.C


EZn.
H
O
H
O
NADH
EZn.
NAD+

Scheme 2


Zn
H
O
Ser 48
R
His 51
N
NAD+
pKa 9.6
pKa 7.6
H+

In all cases, the reverse step, the oxidation is feasible. The occurrence of hydroxyl groups bound to 4 and 5 coordinate zinc at biological pH's has some precedence. For instance, Woolley[28] has shown that five coordinate mono aquo species can have a pK_a of around 8.5. The involvement of a bound hydroxyl to four coordinate zinc with a pK_a of around neutrality has been proposed in carbonic anhydrase action.[29] Since alcohols have similar pK_a's to water the same behavior may be expected. Eklund[1b,26] et al have proposed a mechanism by which a hydrogen relay system could stabilize a bound hydroxyl (Scheme 2) and which allows for the loss of proton into the bulk solution.

Dunn[30,31] has demonstrated spectrophotometrically that the chromophore 4-N,N-dimethylaminocinnamaldehyde is coordinated to zinc and that this species is involved in the reduction process. An X-ray determination,[30] to 4.5 Å resolution, supports this coordination, the complex appears to be four coordinate thereby favoring mechanism A. It was found that the reduction rate was independent of pH.[30] Based on this and other evidence, it was concluded that the protonation step was separate from, and succeeded the hydride transfer.[30]

Additional support for the coordinated substrate comes from studies on the reductions of p-substituted benzaldehydes[20,32,33,34] and oxidation of benzyl

alcohols.[20,34-35] These studies showed small electronic effects for the benzaldehyde reduction whilst for benzyl alcohols the substituent effects were negligible. These results are consistent[32,36] with a bound substrate mechanism in which the carbonyl is polarized considerably by coordination to the zinc ion. The resultant complex in which there is a substantial positive charge on the carbonyl carbon is thus close energetically to the transition state, hence the reaction is relatively little affected by substituents.

An alternative explanation for the lack of substituent effect with benzyl alcohol oxidation is that the hydride and proton transfer steps are concerted[20] and for this reason mechanism B, which includes the presence of a proton source at the active site (the water molecule) was proposed.[20] The proton relay system of Eklund[1b,26] was included to allow for stabilization of the bound hydroxyl.

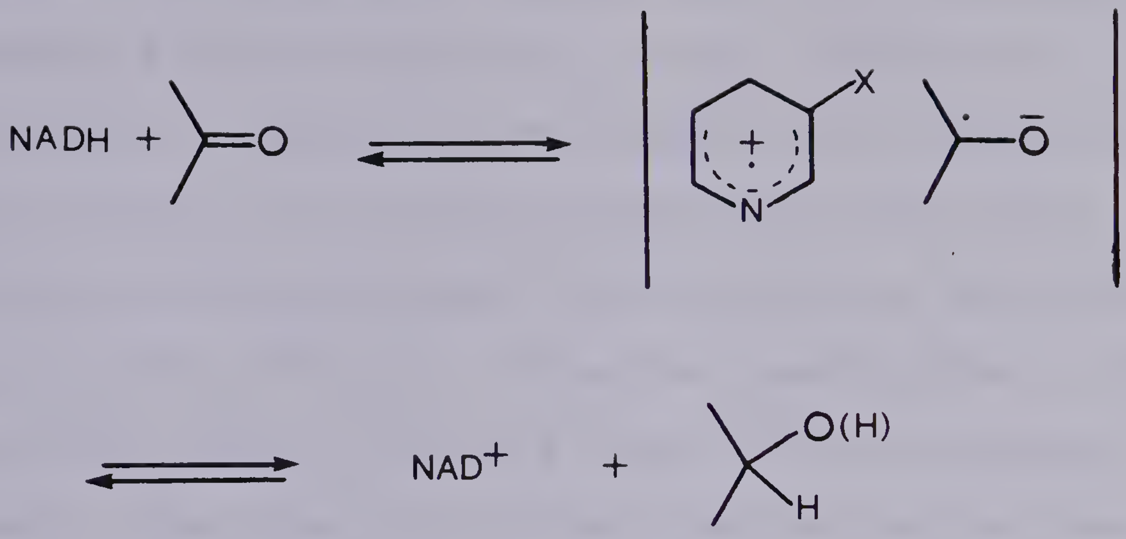
A five coordinate zinc mechanism was supported by McFarland et al[37] who showed, by the absence of a pre-equilibrium isotope effect that water was not expelled on binding of aldehyde. The deuterium isotope effect for the reduction reaction was found to be unity indicating that the protonation was not concerted with the hydride transfer, the reaction mechanism proposed was thus amended accordingly.

Klinman[38] has shown that for benzaldehyde reduction, catalyzed by YADH, a single functional group (pK_a = 8.25), which must be protonated for the reduction reaction and deprotonated for the reverse, is present. Other authors have come to similar conclusions that some ionizing group(s) is involved in the catalytic redox process.[20,39] This ionizing group has been proposed to be bound water (or alcohol), imidazole or cysteine, of which the first is by far the most popular, though it is uncorroborated by any concrete evidence.

The third mechanism (C) was proposed by Sloan[27] based on NMR studies of cobalt HLADH. Calculations showed that ethanol cannot approach within 6 Å of the metal ion and so the bridging water mechanism was formulated.

Finally mention must be made of the single electron transfer (SET) mechanism. This route involves separation of the hydride transfer into two steps: initial electron transfer followed by hydrogen atom transfer (Scheme 3). No definite evidence has been presented for an electron transfer route occurring, however, this does not preclude such a mechanism since the two steps of the process may be almost simultaneous and difficult to separate, c.f.the Grignard reaction with ketones.[40]

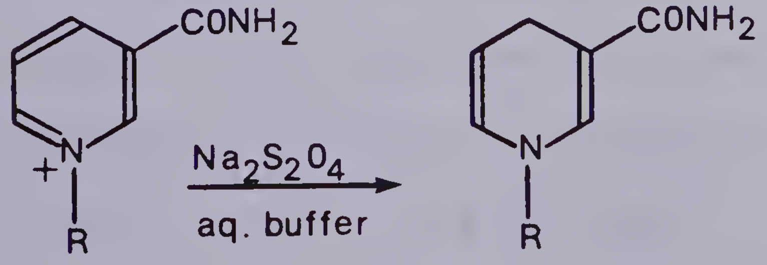
Scheme 3


NADH
O
X
N
Ō
NAD+
O(H)
H

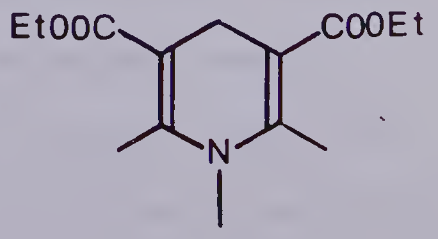
Previous model studies

As has been discussed previously, the zinc ion at the active site of the enzyme has been proposed to act as a Lewis acid catalyst for the reduction reaction. Several models have been reported which test this hypothesis and it has been shown that the presence of metal ion can influence the rate of reduction of a coordinated carbonyl.

Since NADH itself is a rather expensive and complex molecule, several cheap and readily available substitutes have been used. For instance, the NADH analogue 2 and "Hantzsch ester" 3 have been found to be effective

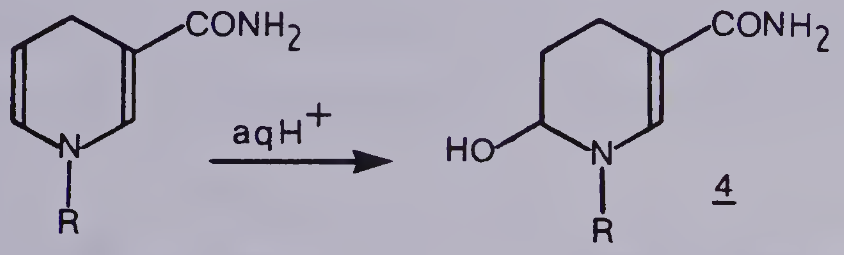

CONH$_2$
N
R
$Na_2S_2O_4$
aq. buffer
CONH$_2$
N
R

1 2


EtOOC
COOEt
N

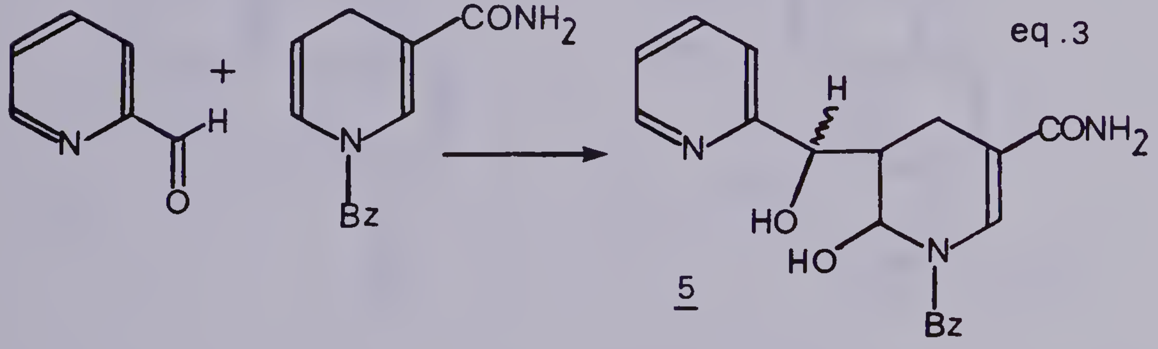
3

reducing agents in model systems. For the NADH analogue 2 (prepared by dithionite reduction of 1[41]), the usually encountered R substituents are *n*-propyl (PNAH) and benzyl (BNAH). Although these compounds are stable in aqueous solutions at high pH's they suffer from acid catalyzed hydration, (as does NADH itself), at lower pH's giving hydrates 4[42] (eq. 2). A further complication is the formation of adducts such as 5[43] (eq. 3) with carbonyl compounds. The formation of these derivatives has been



4

eq.2



5

eq.3

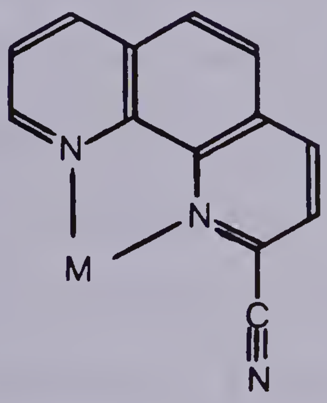
recently proposed to complicate kinetic and isotope analyses[44].

This discussion of models will be divided into two sections. The first part will deal with two examples illustrating the role of electrophilic metal catalysis in reactions involving nucleophilic addition to multiple

bonds to carbon. The second part will show some of the successful models for the reduction reaction that have been reported.

The rate of hydration of 2-cyano-1,10-phenanthroline[45] is increased by a factor of 10^7 and 10^9 in the presence of Ni^{++} and Cu^{++} respectively. Both of these metals are strongly bound by the ligand, presumably in the manner shown (6). An external attack by hydroxide was preferred to a bound nucleophilic hydroxyl by studies with other nucleophiles although in principle, the two mechanisms are kinetically indistinguishable. Since the nitrile cannot coordinate to the metal ion, a transition

6



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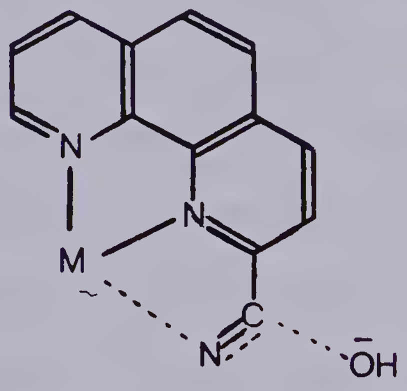
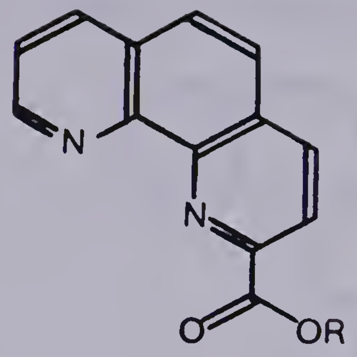


state (7) was proposed in which the incipient negative charge on nitrogen was stabilized by the metal ion.

The effect of metal ions on the rate of hydrolysis of ester 8 has been investigated.[46] The metal ion will bind to 8 analogously as in 6 and may be further coordinated by the ester carbonyl. Large rate enhancements were found over the uncatalyzed case for the metal



8

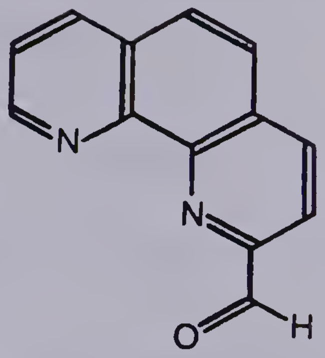
complexes (3000 times for zinc and 80000 times for copper) thus illustrating the metal ion catalysis of nucleophilic addition to coordinated C=X bonds.

As mentioned before, the value for the equilibrium constant for eq. 1 lies heavily in favor of the reduction reaction and indeed most of the model studies reported have dealt with this process.

Considerable work has been done on the reduction of pyridine and phenanthroline carbonyl species in anhydrous acetonitrile and it has been shown that the rates of reduction of the carbonyl are enhanced in the presence of metal ion (see later). Since the product of such a reaction is the alcoholate and not the alcohol it is a debatable point as to whether these studies are directly related to the ADH system. Certainly, no information can be gained about the question of whether the hydride and proton transfer steps are concerted, however valuable insight into the catalytic role of zinc has been obtained (see later).

The reduction of 1,10-phenanthroline-2-carboxy-

aldehyde 9 by PNAH in anhydrous acetonitrile has been



9

investigated.[47,48] No reaction at all occurs in the absence of metal but with zinc ion present (2 eq.), the corresponding alcoholate is formed in very high yield. Using the 4,4-dideuterated reducing agent, mass spectral analysis indicated the transfer of one deuterium into the product, as predicted. A kinetic isotope ratio, k_H/k_D of 1.7 ± 0.6 was seen indicating that the hydrogen transfer is involved in the rate limiting step. To test that the rate enhancement is indeed caused by direct coordination of the carbonyl to zinc rather than a purely electronic effect caused by nitrogen coordination, the reduction of zinc complexes of 2- and 4-pyridine aldehydes by tetraethylammonium borohydride was investigated. It was found that the zinc complex of the 4-isomer was reduced 100 times faster than the uncomplexed form. For the 2-isomer an enormous rate enhancement (700000 times) was seen. Whereas bidentate coordination of the 4-isomer is impossible, the data for the 2-isomer indicate that such may be occurring. Kinetic analysis reveals that the two isomers are reacting via different

mechanisms. For the 4-isomer, simple bimolecular reduction is occurring:

$$M + A \rightleftharpoons MA$$

$$MA + BH_4^- \longrightarrow \text{product}$$

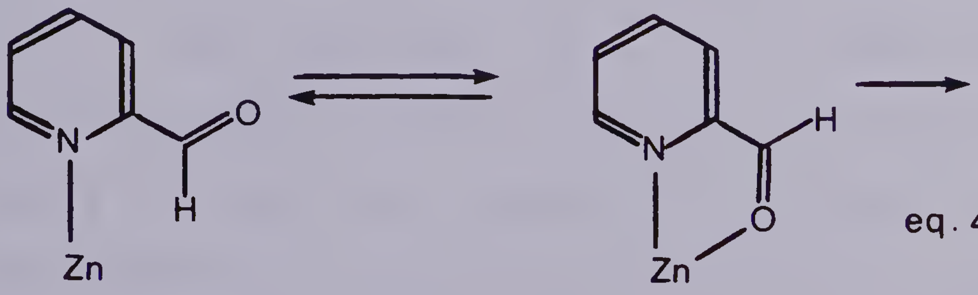
For the 2-isomer, the rate is independent of borohydride concentration so a second route was proposed:

$$M + A \rightleftharpoons MA$$

$$MA \underset{}{\overset{\text{slow}}{\rightleftharpoons}} MA^*$$

$$MA^* + BH_4^- \xrightarrow{\text{fast}} \text{product}$$

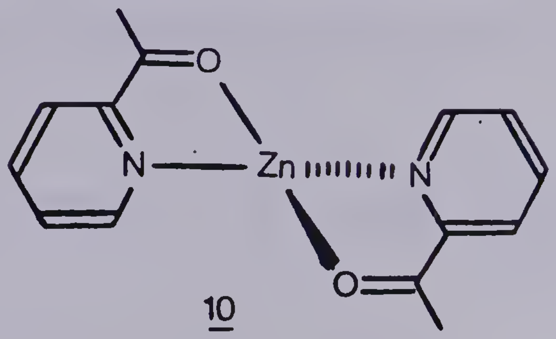
An attractive explanation is that the initial complex, MA, is in the E form which isomerizes in a slow process to give the Z form MA^*, which is coordinated to zinc and readily reducible eq. 4.



eq. 4

Whereas 2-acyl pyridines exist predominantly as the E form in the liquid phase[49] it has been demonstrated that bidentate coordination is possible. Some 2:1 complexes of 2-cinnamoyl pyridines with zinc have been isolated[50] and reveal that the carbonyl is a chelating ligand,

giving octahedral or tetrahedral complexes e.g. 10, depending on counterion.



10

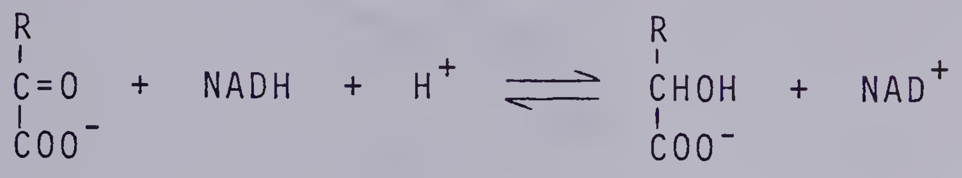
It was found that although in the absence of metal ion no reduction occurred, in the presence of zinc or magnesium (1 eq.) in dry acetonitrile, conjugate reduction occurred. The 3- and 4-isomers were also reduced though at a much slower rate.

Pandit et al[51] showed that in the presence (but not in the absence) of zinc or magnesium ions, 2 benzoylpyridine was reduced in acetonitrile by BNAH whilst the 3- and 4-isomers were inert. In similar work Ohno et al[52] showed that 2-acetyl pyridine was also a substrate. In this work it was found that the rate of reduction depended on the ratio of ketone to zinc and it was concluded that the 2:1 ketone:zinc complex was the most active.

The reduction of pyridine-2-aldehyde has been studied in methanolic solution and a rate enhancement for reduction in the presence of Zn^{++}, Pb^{++}, Co^{++} and Cu^{++} was seen.[53]

A distinction must now be made between the action of alcohol dehydrogenase and lactate dehydrogenase

(LDH).[54] LDH's catalyze the reduction of α-keto acids to α-hydroxy acids using NADH/NAD$^+$ (eq. 5). Whilst the redox reaction appears to be similar to that in ADH



$$\begin{array}{c} R \\ | \\ C=O \\ | \\ COO^- \end{array} + NADH + H^+ \rightleftharpoons \begin{array}{c} R \\ | \\ CHOH \\ | \\ COO^- \end{array} + NAD^+$$

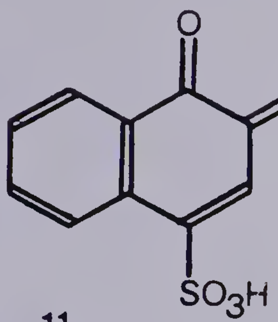
eq. 5

action (eq. 1), this enzyme contains no zinc ion at the active site. Some model studies for the reduction of α-keto carbonyl compounds do include the use of metal ions to effect reaction and so will be included in this discussion.

The reduction of α-keto carbonyl compounds has been investigated by Ohnishi and Ohno in anhydrous acetonitrile.[54-56] The reductions are catalyzed by magnesium perchlorate[54-56] but not lithium perchlorate,[54,56] are not retarded by hydroquinone,[54,56] but are hindered by the presence of water.[54,56] In one case,[54] a diketone was successfully reduced to the diol though in low yield. The use of a chiral NADH analogue has lead to asymmetric induction in the reduction product.[55,56]

A polymer, containing pyridine (to complex zinc) and dihydronicotinamides was found to reduce benzil to benzoin in aqueous ethanol.[57] With zinc or nickel ions present, the rate of reduction was slightly enhanced (≤ 50% increase over the uncatalyzed case).

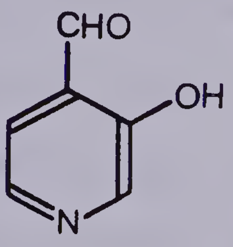
The reactive naphthaquinone derivative 11[58] can be



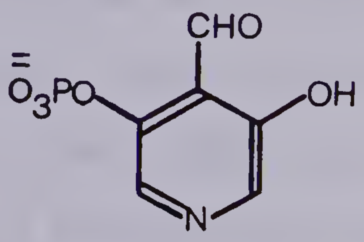
11

reduced by NADH in methanol, introduction of zinc sulphate heptahydrate increasing the rate of reduction of 11 by an order of magnitude. In the presence of the zinc complexing agent, 1,10-phenanthroline the rate of reaction was lowered.

Shinkai and Bruice have studied the reduction of some pyridine 4-aldehyde species in aqueous methanol.[59,60]



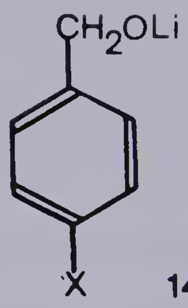
12



13

It was found that reduction of 12 by PNAH and 13 by PNAH and a Hantz ester was accelerated by the presence of Ni^{++}, CO^{++}, Zn^{++}, Mn^{++} and Mg^{++}. Although the rate enhancement appeared to be modest (k_{rel} for the nickel case 7.2, compared to the uncatalyzed reaction), however the work was carried out in EDTA buffer and so the catalysis may in reality have been somewhat larger if

metal ion had not been sequestered away.



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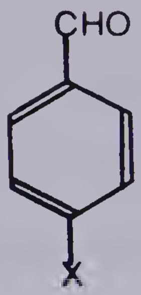


15

A model for the oxidation reaction was reported by Shirra and Suckling[61] who showed that the lithium alkoxide 14 reacted with a NAD$^+$ analogue to give an equilibrium mixture with the oxidized product 15.

No definite evidence from models has been put forward to support the electron transfer (SET) route. In several cases the effect of added hydroquinone on the rate of reduction in model systems was investigated but in no case was any change seen. (Should the SET proceed via an intimate caged pair, rather than free radicals it is unlikely to be affected by hydroquinone). van Eikeren et al[62] have recently proposed that the reaction of PNAH and pyridyl ketones proceeds via a caged radical pair intermediate (see Scheme 3) in aqueous solution, however this work was carried out in the absence of metal ion and may not be relevant for ADH action.

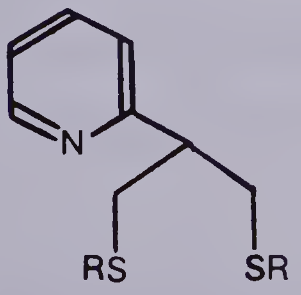
Scope and purpose of this research

Convincing evidence has been presented concerning

the role of zinc as an electrophilic catalyst during the reduction reaction (vide supra). This research is concerned with the synthesis and study of small molecules which specifically mimic the ADH active site and can be modified to test the hypothesis of electrophilic catalysis of carbonyl reduction in the presence of the known Zn^{++} coordinating groups.

No model studies to date have attempted to approximate the known active site of the enzyme, i.e. 2 thiols and 1 imidazole nitrogen as ligands to zinc. The following report will describe the construction and physicochemical properties of novel chelating systems which mimic the ADH Zn^{++} binding site.

Two systems were chosen to mimic the binding site, one based on pyridine and the other on imidazole, both as appendages on a 1,3-dithiopropane skeleton as shown in 16 and 17, where R could be aryl, alkyl or hydrogen.



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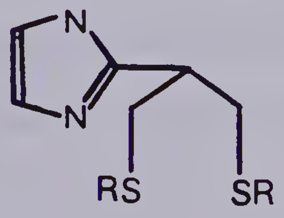
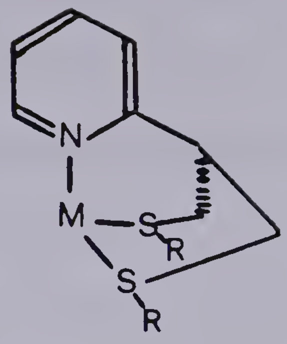
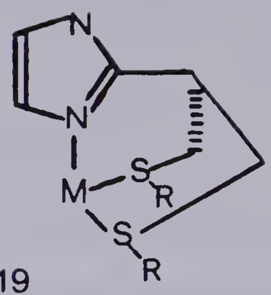


17

If coordination through all three ligating atoms occurs then complexes of the form 18 and 19 would be expected with metals.



18



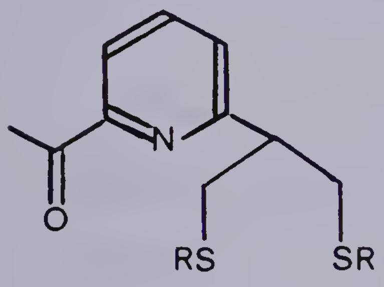
19

Since these compounds are new, their abilities to bind metal ions are unknown and therefore must be established. Pyridine and imidazole are rather weak ligands by themselves,[63] however the introduction of a second ligating group into the molecule increases the binding considerably (e.g. for Zn^{++}, pK_L[64] for pyridine ≈ 1 and for 2,2'-bipyridyl ≈ 5[63]). It is known that thiols bind zinc ion strongly[63] so it can be reasonably assumed that the dithiol forms of 16 and 17 will be tightly bound. Thioethers, however, are only modest ligands[63] to zinc and it may be expected that these forms of 16 and 17 will be less well bound than the former. However, it is anticipated that the chelate effect[65] could give rise to reasonably stable complexes of the latter.

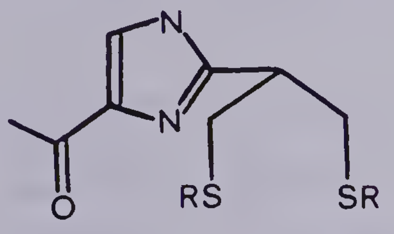
The second part of this report describes the introduction of a carbonyl group to the binding site and describes the general synthesis of 20 and its analogues. The analogous imidazole compound 21 is not considered suitable since the carbonyl is a vinylogous amide and

thus should not be reactive. The ketone derivative was chosen in place of the aldehyde since the latter are prone to metal catalyzed hydration.[66]

The electrophilic hypothesis can now be tested in two ways. By analogy with 18, 20 may be expected to bind zinc as in 22, the carbonyl being drawn as the Z form, the mode by which the reduction is expected to take place. The actual equilibrium conformation does not necessarily have to be that of the Z form as long as free rotation can occur to give this species. If binding does occur as shown, then by varying the electronic nature of R, where R is a substituted aryl, correlations may be made between carbonyl reducibility (if any) and the electron demand on zinc.



20



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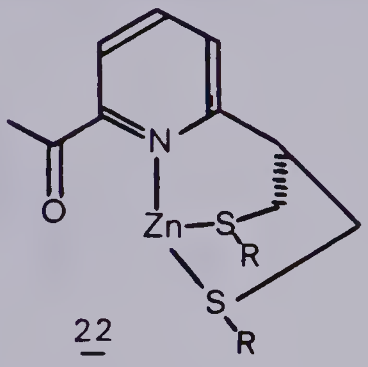
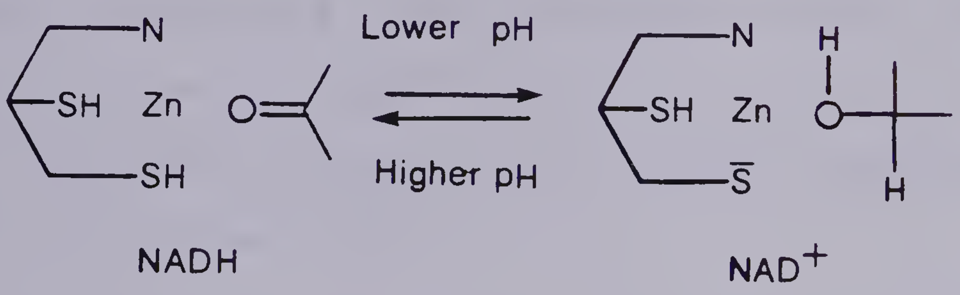


22

The second approach to testing the hypothesis is best explained by returning to the enzyme itself. An ionizing group, at, or near the active site has been shown to affect the enzyme action (vide supra). This has been mostly proposed to be the bound water, however, simple examination of the acidities involved reveals

alternative candidates. For instance, whereas water has a pK_a of 15.7, thiols have pK_a's of around 10 in aqueous solutions so on purely electrostatic grounds a bound thiol would be expected to be more acidic than bound water. Whatever the actual ionizing group involved, the situation depicted in eq. 6 would be expected above



eq. 6

and below its pK_a (with thiol deprotonated for clarity). At low pH's the zinc retains an effective 2+ charge but at higher pH this is reduced to a net unipositive charge and so reducing its electrophilic capabilities so that at higher pH the reduction might be less favored. Thus at higher pH, the oxidation of alcohol is favored since the zinc is no longer electrophilic. Of course, from the equilibrium constant given in eq. 1, ones sees that the position of equilibrium is determined by $[H^+]$. However, the rate of the catalyzed reduction or oxidation reaction is likely to be sensitive to the electropositive character of the metal which can depend on the state of ionization of the Zn^{++} ligands.

RESULTS AND DISCUSSION

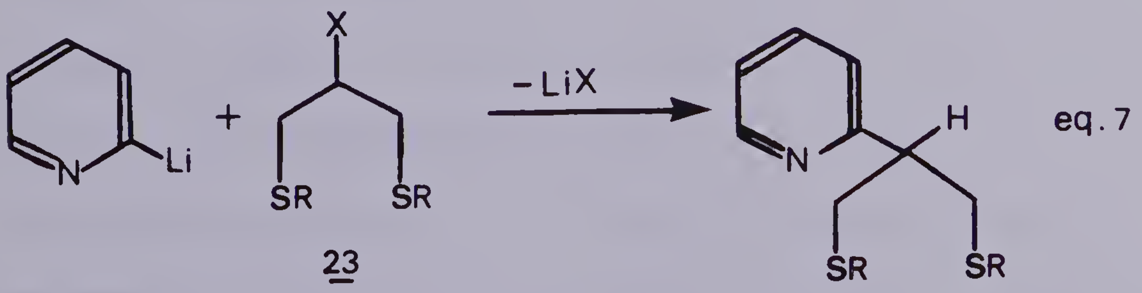
Introduction

Since the compounds studied as enzymes models represent an unknown class of ligands, their syntheses will be discussed in detail. The order of contents in this section will be purely chronological, starting with ligands containing a basic nitrogen and two thioethers, continuing with ligands containing a basic nitrogen and two thiols and finishing with ligands containing a basic nitrogen and one thiol.

Ligands containing a pyridine and two thioether groups.

i. Synthesis

Two synthetic schemes to prepare the title compounds were attempted, both involving the reaction of 2-lithiopyridine (readily prepared from n-BuLi and 2-bromopyridine)[67-9] with a 1,3-dithiopropane species, containing an electrophilic centre at C_2.

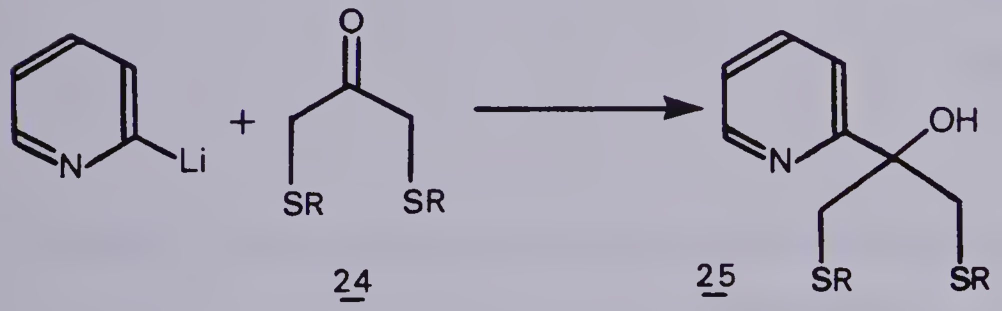


The procedure outlined in eq. 7 was found to be unsuccessful because of difficulties encountered in pre-

paring the reactive compounds of form 23. Tosylates of 23 (X = Tos, R = CH_3 or C_6H_5) could not be prepared although the parent alcohols were readily available, and an iodide (R = CH_3, X = I) was found to be unstable at room temperature.

The coupling scheme depicted in eq. 8 was found to proceed, albeit in moderate yields, though only if an excess of 2-lithiopyridine to ketone 24 is used. For instance, no product (25) at all was observed when a 1:1 ratio of 2-lithiopyridine with 24f was tried. However, with an excess of the lithium reagent the adduct was formed, though even with a 3:1 ratio the yields were modest ($\leq$ 42%). The reasons for this behaviour are not clear. 2-Lithiopyridine gave a 50% yield of the adduct with acetone when a 1:1 mixture was tried and both methyl magnesium chloride (with 24e) and phenyl magnesium bromide (with 24b) gave similar NMR yields in 1:1 ratios.

eq.8



24

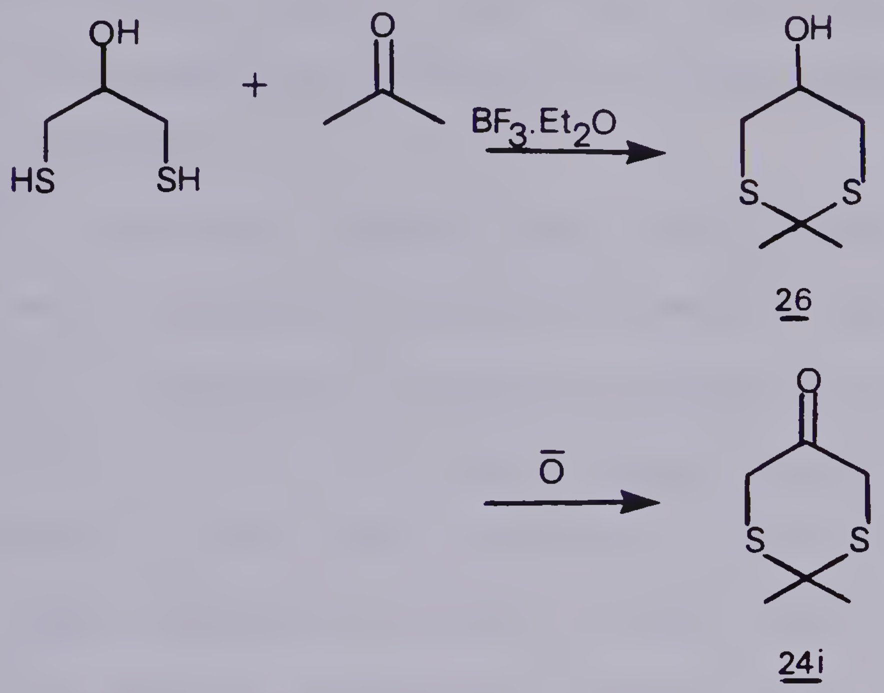
25

(24,25)	R
a	CH_3
b	C_2H_5
c	4-$Me_2NC_6H_4$
d	4-$MeOC_6H_4$
e	4-MeC_6H_4
f	C_6H_5
g	4-ClC_6H_4
h	3-$CF_3C_6H_4$

The dithio ketones 24a-h were prepared by the

double displacement of 1,3-dichloropropan-2-one by the sodium salt of the appropriate thiol.[70]

The cyclic ketone 24i[71] was prepared as in eq. 9.



eq. 9

Although the formation of 26 proceeded in almost quantitative yield, the oxidation step (Moffatt or lead (IV) acetate/pyridine[72] procedures) was found to proceed only in low and erratic yields. Adduct 25i was prepared from 2-lithiopyridine and 24i.

ii. Physical studies

Ionization constants (pK_a's) of the protonated ligands were determined for thioethers 25a-i and 2-(2'-pyridyl)-propan-2-ol[73] (27) at concentrations of 4 x 10^{-3} M in 50% (v/v) aqueous dioxane at 25°C and constant ionic

strength of 0.3 M $NaNO_3$. The data were analyzed by a computer version of Simms' method[74] and are given in Table I. A general trend emerges when one compares the pK_a's of the substituted aryl thioethers 25d-h; electron withdrawing groups on the phenyl ring reduce the pK_a's in this medium substantially, as expected on inductive grounds.

pK_L[64] values for ligands 25a-i and 27 with Co^{++}, Ni^{++} and Zn^{++} were determined by potentiometric titrations on 5 x 10^{-3} M solutions of ligand containing a 20-fold excess of metal ion in the same medium as before. Data were analyzed by the method of Martell and Calvin.[75] For the copper complexes, where the pK_L values are larger, the method described by Huguet[74] was used.

On the basis of the data in Table I, one can see that ligands 25a-i bind the divalent ions weaker than does 27. Thus it appears that the dithioether functionality does not contribute constructively to the complex formation. Comparison of the data for 27 with those for pyridine[75] and 2-picoline[75] shows that 27 binds the divalent ions more tightly, probably by involvement of the hydroxy group as in 28. Since, in general, the thioethers bind more poorly than 27 but better than 2-picoline we cannot tell whether their chelates resemble 28 or 29 although one notes that the electron-withdrawing groups on the aryl ring decrease the binding ability

TABLE 1. pK_a's and pK_L's for ligands 25, 27 and related compounds (see text for details).

Compound	pK_a[a,b]	pK_L[a,c]			
		Co++[d]	Ni++[e]	Cu++[f]	Zn++[g]
25a	3.60	1.21	1.82	3.08	1.23
25b	3.61	1.17	1.60	3.13	1.20
25d	3.54	0.84	1.22	-	1.05
25e	3.47	0.86	1.18	2.92	0.86
25f	3.37	0.81	1.11	3.01	0.88[h]
25g	3.16	0.66	0.80	2.84	0.83[h]
25h	2.98	0.45	0.50	2.87	0.68
25i	3.22	1.16	1.71[i]	2.70	1.21[h]
27	4.18	1.99	2.56	3.53	1.88
pyridine[75]	5.33	-	1.85	2.54	1.07
α picoline[75]	6.06	-	<1	1.3	<1

a. Obtained by potentiometric titration of 4-5 x 10^{-3} $\overline{M}$ solutions of compound in 50% (v/v) aqueous dioxane at 25°C and I = 0.3 ($NaNO_3$).

b. Precision ± 0.01 unit. Reported values are the averages of 3 determinations.

c. Each value is the average of three determinations and has a precision of ± 0.06 units or better.

d. 0.0904 M.

e. 0.0812 M.

f. Determined as in ref. 74.

g. 0.0854 M.

h. 0.0867 M.

i. 0.0406 M.

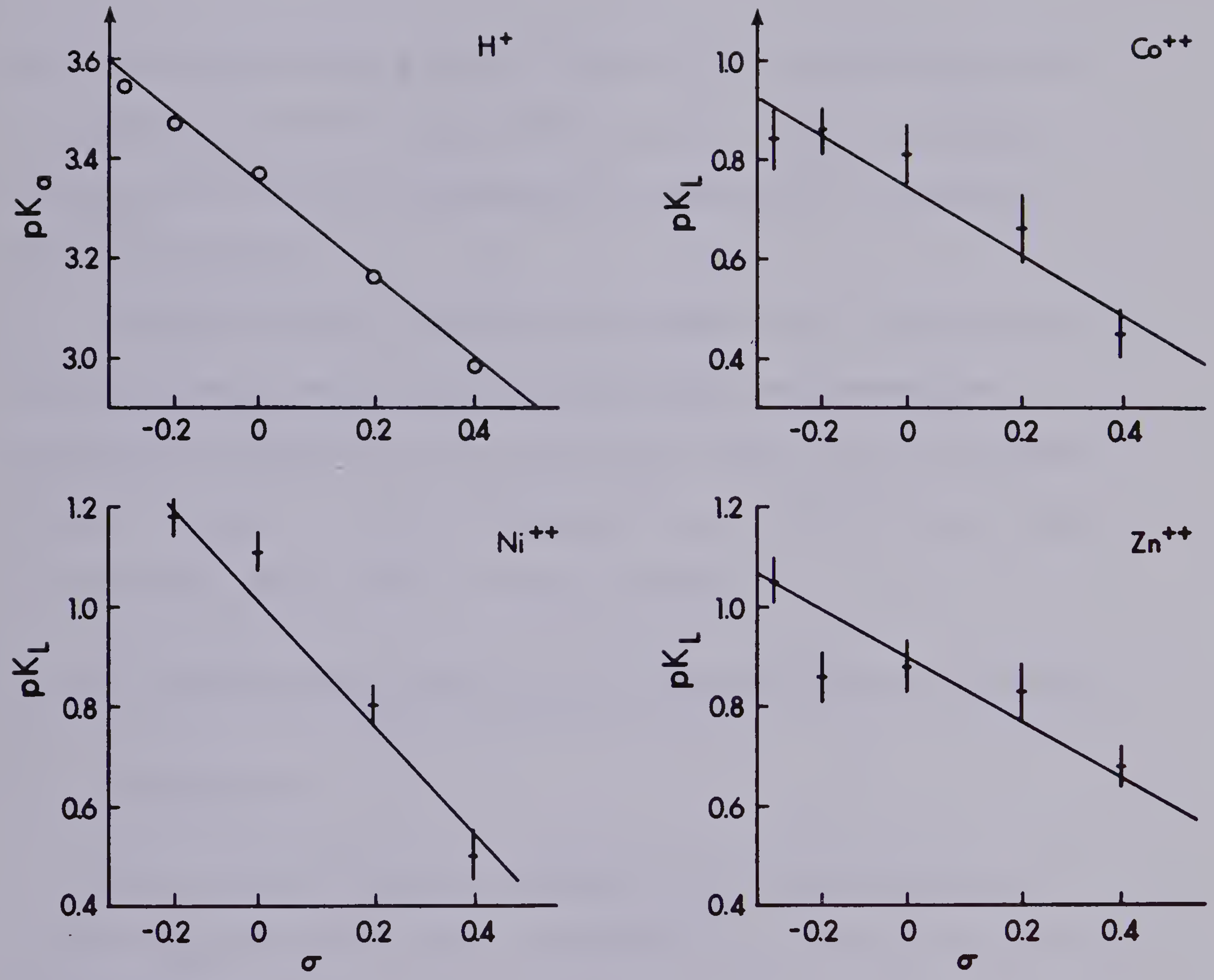
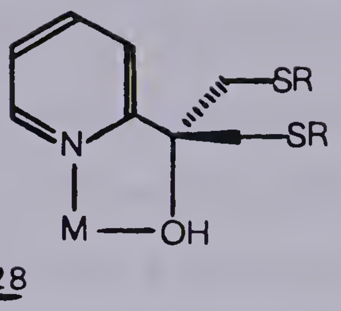
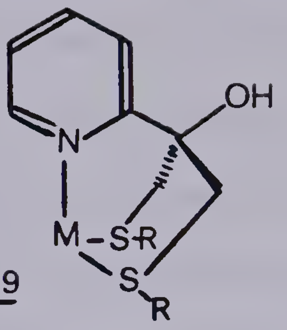


Fig. 2.

Plots of pK$_a$ and pK$_L$ against σ for 25d-h.


28


29

as they do the pK_a's (see Table I) (A correlation with σ is seen in both cases, see Fig. 2). Thus probably a similar inductive mechanism is operative in both H^+ and M^{++} binding.

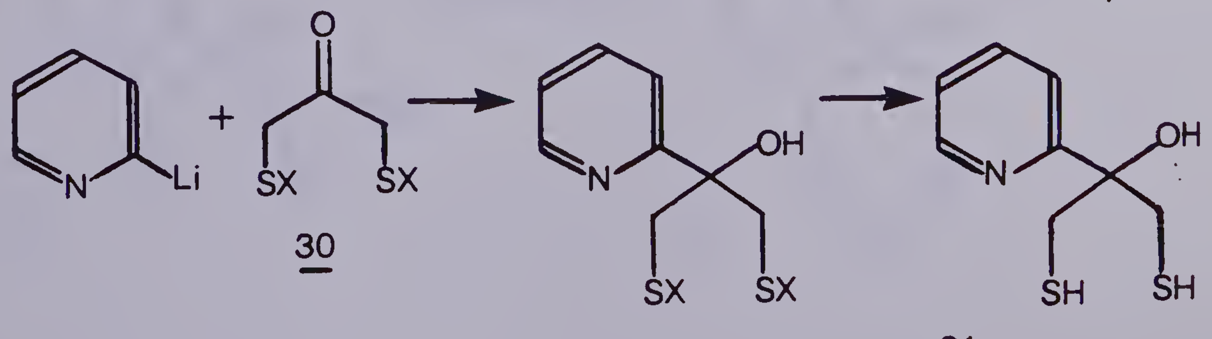
These results indicate that whereas it seems possible to vary metal binding affinity and therefore electronic demand on zinc to some extent, the thioether ligand system is unsatisfactory for further study since it does not bind zinc strongly enough.

Ligands containing pyridine or imidazole and two thiols.

i. Synthesis

The method discussed before for the formation of adducts 25 can be readily extended as a route to analogous dithiol ligands.

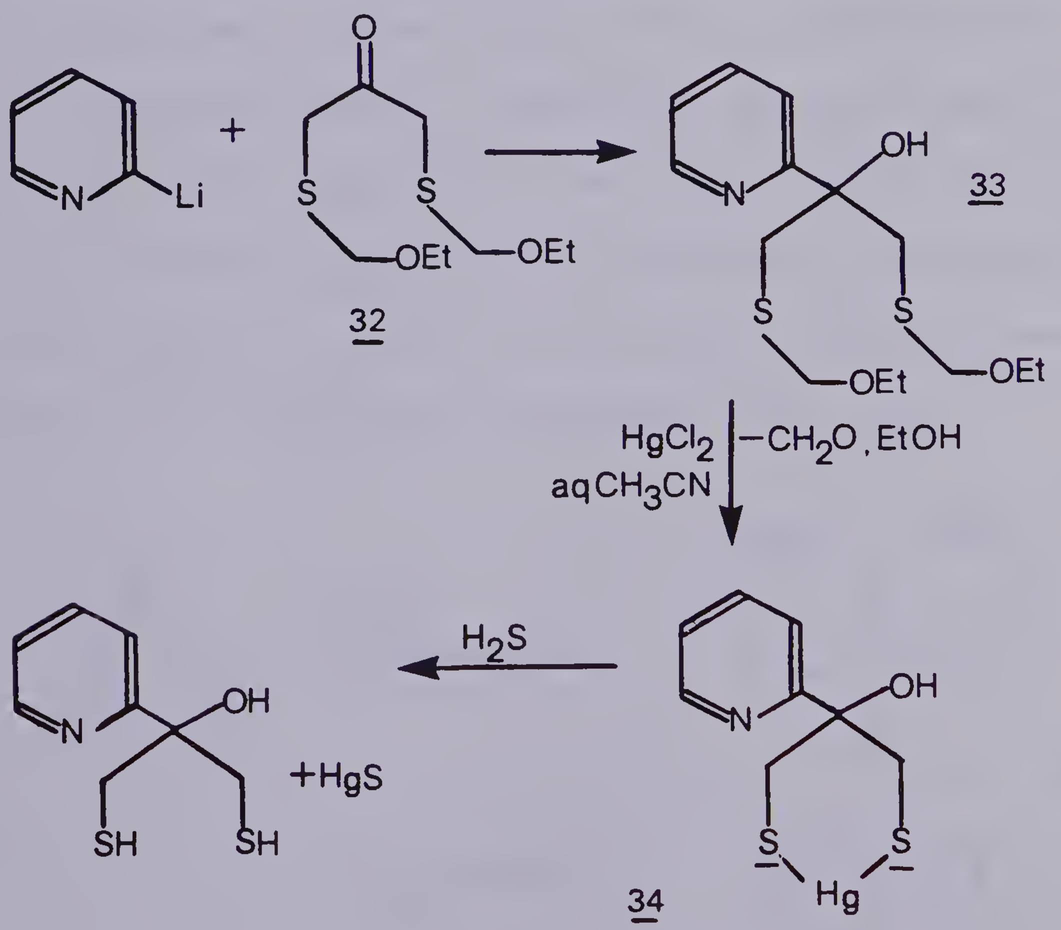
eq. 10


30

31

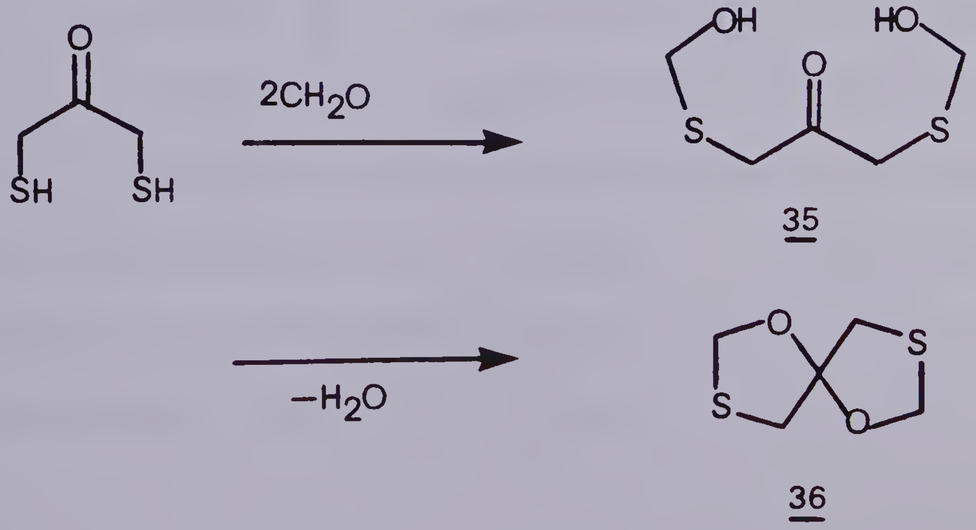
The use of a suitably S-protected ketone 30 would give rise to the adduct as before and subsequent deblocking would give the target molecule 31 (eq. 10). Corey[76] has shown that the $-CH_2SCH_3$ group is an effective protecting group for alcohols which can be readily removed under mild conditions ($HgCl_2$ or $AgNO_3$, in solution at room temperature). Conversely, it is to be expected that the $-CH_2OR$ group can be used to protect thiols, and indeed, this approach lead to reasonable yields of 31 as in eq. 11. Treatment of 2-lithiopyridine with the protected ketone 32 gave the adduct 33 which was isolated in a reasonably pure form by acid

eq. 11


N
Li
+
O
S
S
OEt
OEt
32
OH
33
S
S
OEt
OEt
HgCl2
aqCH3CN
−CH2O, EtOH
H2S
OH
N
SH
SH
+HgS
31
N
OH
S
S
Hg
34

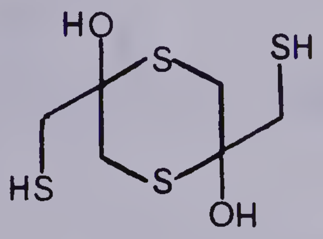
extraction. Addition of a aqueous acetonitrile solution of $HgCl_2$ to the adduct 33 gave an immediate precipitate (presumably 34) and a strong smell of formaldehyde was noticed. It is believed that 34 is deprotonated since the solution became very acidic. Treatment of an aqueous solution of 34 with H_2S gave 31 and a black precipitate of the infinitesimally soluble HgS.[77]

Ketone 32 was prepared by reaction of the 1,3-dilithio anion of "1,3-dimercapto acetone"[78] with 2 eq. of chloromethyl ethyl ether in dry THF. The disodium anion of "1,3-dimercaptoacetone" has been used in aqueous solution to effect S-alkylation by reaction with iodoalkanes, however when this procedure was tried for the present example, two major products were formed. One of these was 32 whilst the other, which had empirical formula $C_5H_8O_2S_2$ was tentatively given the structure 36 on the basis of spectral analysis and the existence of a plausible mechanism (eq. 12).

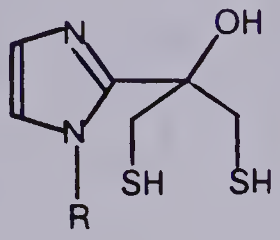


eq.12

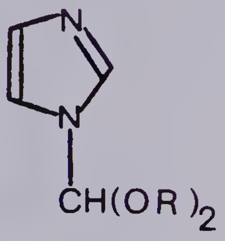
According to Schotte,[78] the melting point of "1,3-dimercapto acetone" is 85-6°C, however in the present work, it was consistently found to be higher (105-9°), therefore meriting further investigation. The product was found to be analytically pure (C,H,N ± 0.3%) and on the basis of NMR, IR and mass spectral evidence, the structure was found to be consistent with the dimeric structure 37.[79]



37



38 R = Me
39 R = H

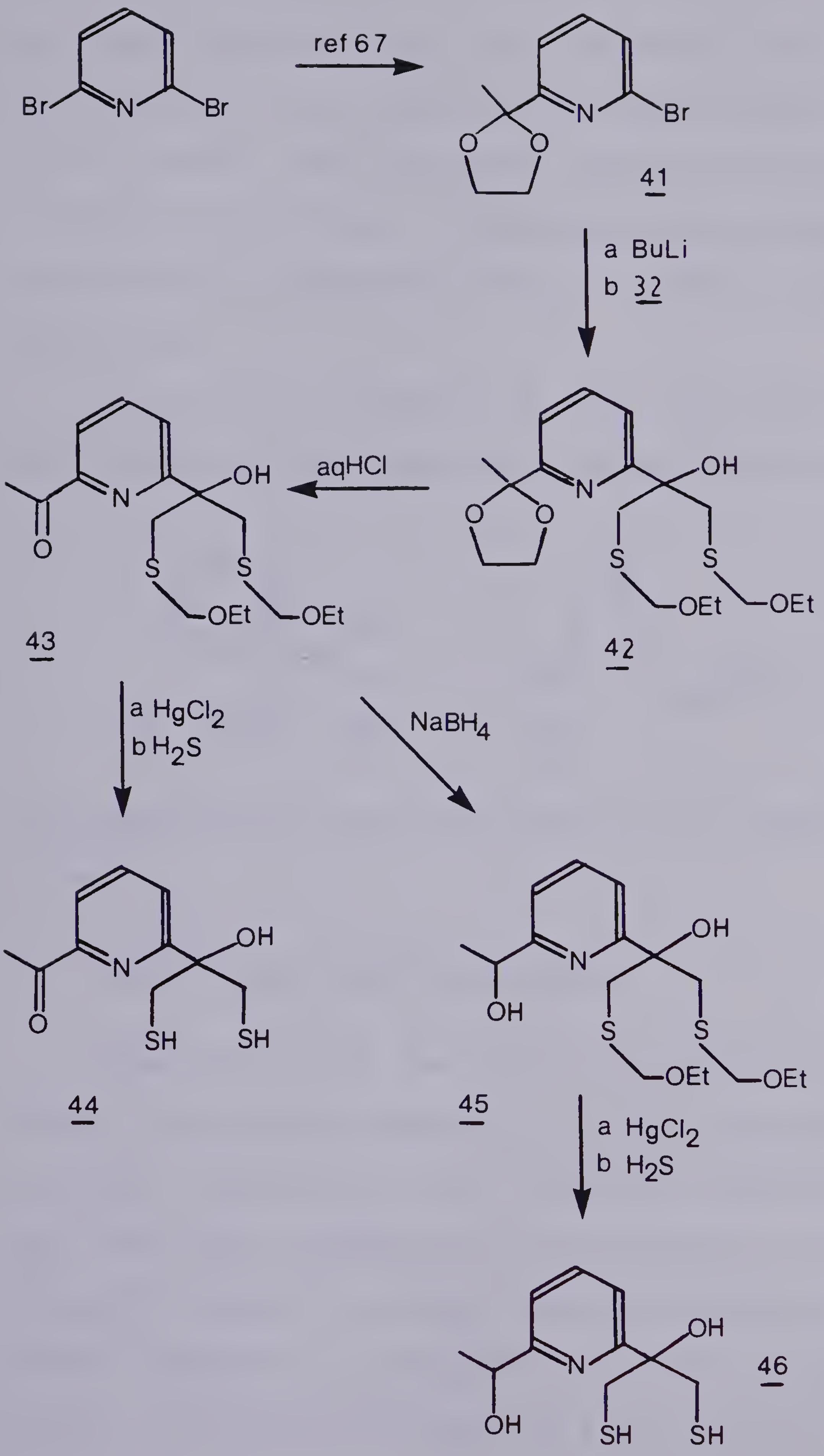


40

The 1-methyl and 1-hydrido-imidazoles 38 and 39 were prepared in an analogous manner to 31, the former from the 2-lithio anion of 1-methyl imidazole and the latter from the 2-lithio anion of the N-protected imidazole 40 (to be discussed in the succeeding chapter).

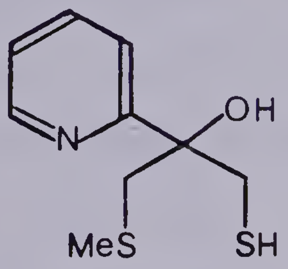
The syntheses of 2,6-disubstituted pyridines containing a ketone (or alcohol) and the dithiol functionality as models of substrate-active site complexes for LADH was achieved as in Scheme 4. Acetylation and protection of 2,6-dibromopyridine gave 41 which was coupled with 32, via its lithium anion, to give the

Scheme 4


Br
N
Br
ref 67
N
Br
O
O
41
a BuLi
b 32
N
OH
O
O
S
S
OEt
OEt
42
aqHCl
N
OH
O
S
S
OEt
OEt
43
a HgCl$_2$
b H$_2$S
NaBH$_4$
N
OH
O
SH
SH
44
N
OH
OH
S
S
OEt
OEt
45
a HgCl$_2$
b H$_2$S
N
OH
OH
SH
SH
46

adduct 42. Adduct 42 was isolated by first extracting the reaction mixture with 0.1 M HCl (to remove debrominated, unreacted 41) and then with 3 M HCl (in which 42 is soluble): on standing, the deprotected ketone 43 was formed. The thioacetal groups were then deblocked as before to give 44. The corresponding alcohol, 45 was prepared by reduction of 43 followed by subsequent deblocking.

In a similar synthesis to that of 31, the monothiol, monothioether 47 was prepared from 48 (made by stepwise



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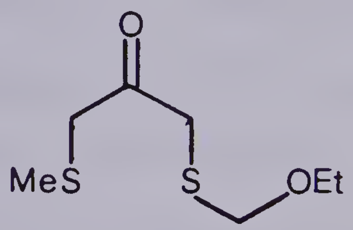


48

ethoxymethylation and methylation of "1,3 dimercaptoacetone").

ii. Physical and reduction studies

Determination of the stability constants for the dithiol containing ligands 31, 38, 39, 44 and 45 is not as simple a task as for the thioethers since precipitation invariably accompanies the titration. Relatively few pK_L[64] values for dithiol complexes have been reported[63] however, the reported values are large. For the Ni-1,2-ethandithiol complex a pK_L of 20.7[63] was

found and for the Zn^{++} complex of 2,3-dimercapto-propan-1-ol a pK_L of 13.5 was found.[63] Utilizing the titration methods used previously for strongly binding complexes,[74] the monothiol compound 47 was found to have pK_L's of 10.8, 10.9 and 10.8 with Zn^{++}, Co^{++} and Ni^{++} respectively. It is therefore expected that the dithiols will also be strong chelators as well, and although reliable pK_L values are not accessible it is believed that they are strongly bound through sulphur.

The reduction of the active site-substrate analogue 44 was investigated. For reasons of solubility of the Zn^{++} complex, the solvent system required to dissolve 3×10^{-3} M in each of 44, $ZnCl_2$ and PNAH was 90% v/v $HMPA/H_2O$. With 2 eq. of added HCl (to make an acid environment) a rapid loss of PNAH was observed by monitoring its absorbance at 350 nm. This was clearly tied to the acid catalyzed hydration of PNAH as was evidenced by a characteristic isosbestic point at 310-5 nm.[42,62] For the same reagents but with 1 eq. NaOH added, a slow loss of PNAH (14% over 21 h) was observed which again was tied to the formation of only the hydrated product. Apparently in this medium we have no evidence to support a Zn^{++} promoted reduction of 44 by PNAH. This seems odd in view of the fact that 2-acetylpyridine and 2-formylpyridine can be reduced by PNAH or BNAH in the presence of added M^{++} ion.[52,53] Apparently the thiol

ligands interfere with the process of reduction.

To investigate the nature and ionization properties of metal complexes with the dithiol ligands, potentiometric titrations were carried out in the absence and presence of metal ion.

As a control experiment, weakly binding thioether 25a was titrated in 50% aq. dioxane (I = 0.3 M, T = 25°C), as the HCl salt, both in the absence and presence of one eq. of metal ion. Figure 3 shows the titration curves and it is seen that the pyridine-H^+ ionization is perturbed only slightly when Zn^{++} is added. It is important to note that for the latter titration only one eq. OH^- is consumed up to a pH ≈ 6: further addition of OH^- resulted in precipitation of $Zn(OH)_2$.

On the other hand, similar titration experiments performed on pyridine monothiol 49 (vide infra), showed (Fig. 4) in the absence of metal, a pK_a of 4.21 corresponding to the pyridine→pyridinium titration and a thiol ionization at pH ≈ 10. In the presence of Zn^{++} however, the titration curve is markedly displaced with 2 eq. of OH^- being consumed by pH 6. At pH's in excess of 6.5, visible precipitation occurred which was tied to the consumption of additional OH^-. For best comparison of the data, it is clearly desirable to perform the titrations on pyridine dithiol 31, but its metal complex precipitated even at low pH. However, the analogous

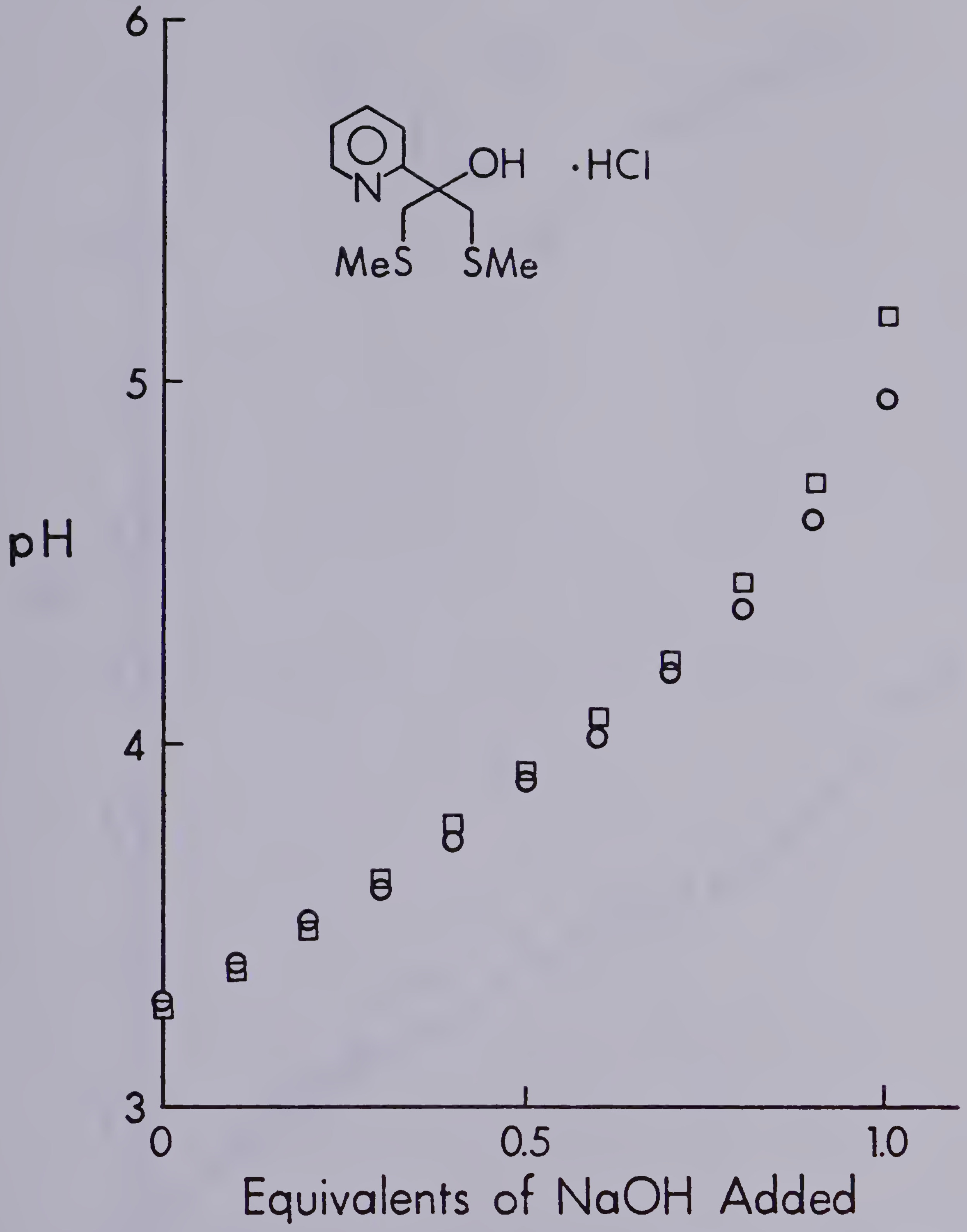


Fig. 3.

A plot of the titration curve for 25a (pH vs equivalents of NaOH added) in the absence (□) and presence (○) of one eq of Zn^{++} in 50% aq. dioxane, I = 0.3, T = 25°C.

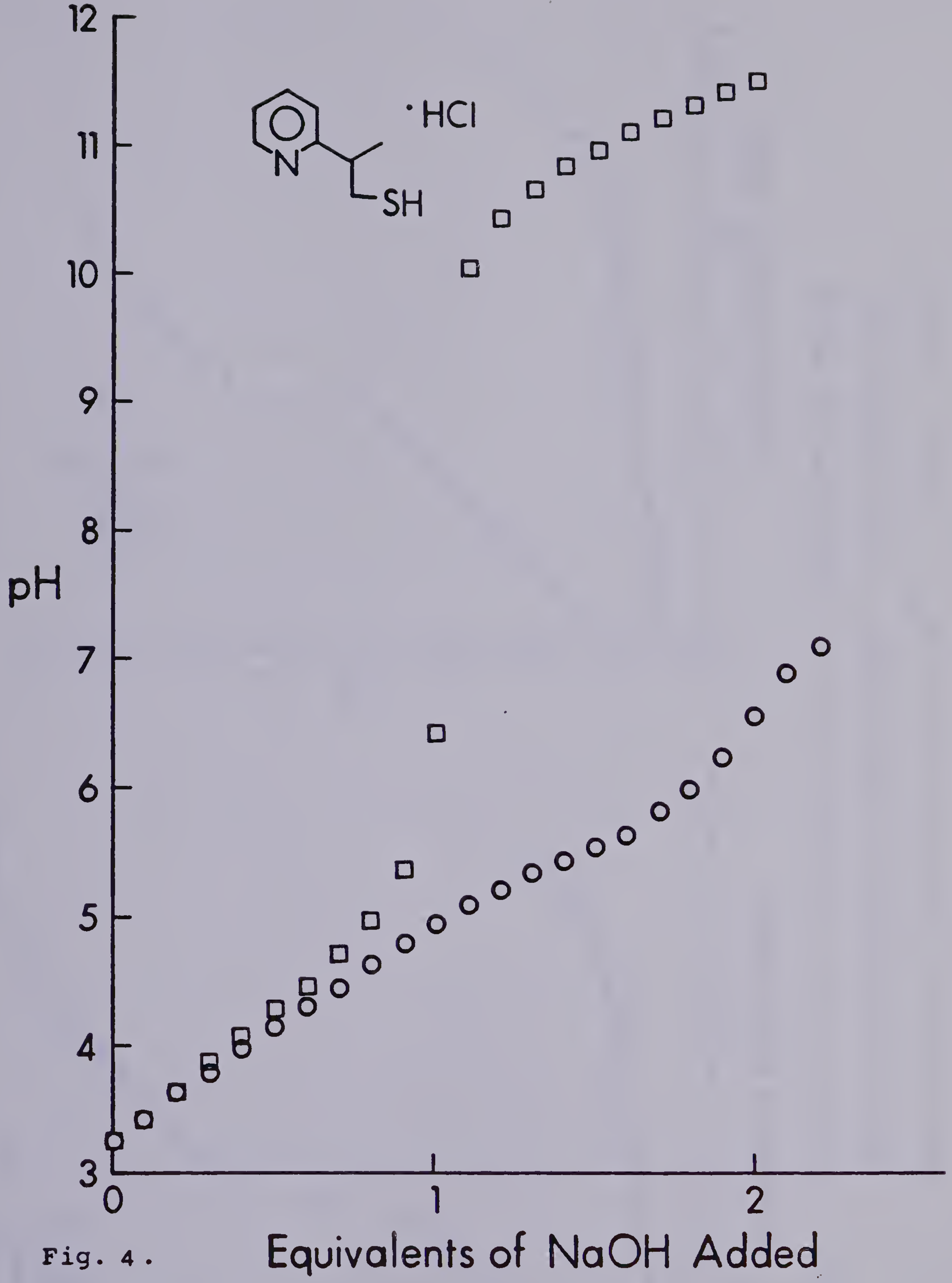


Fig. 4.

A plot of the titration curve for 49 (pH vs equivalents of NaOH added) in the absence (□) and presence of one eq. of Zn^{++} in 50% aq. dioxane, I = 0.3, T = 25°C.

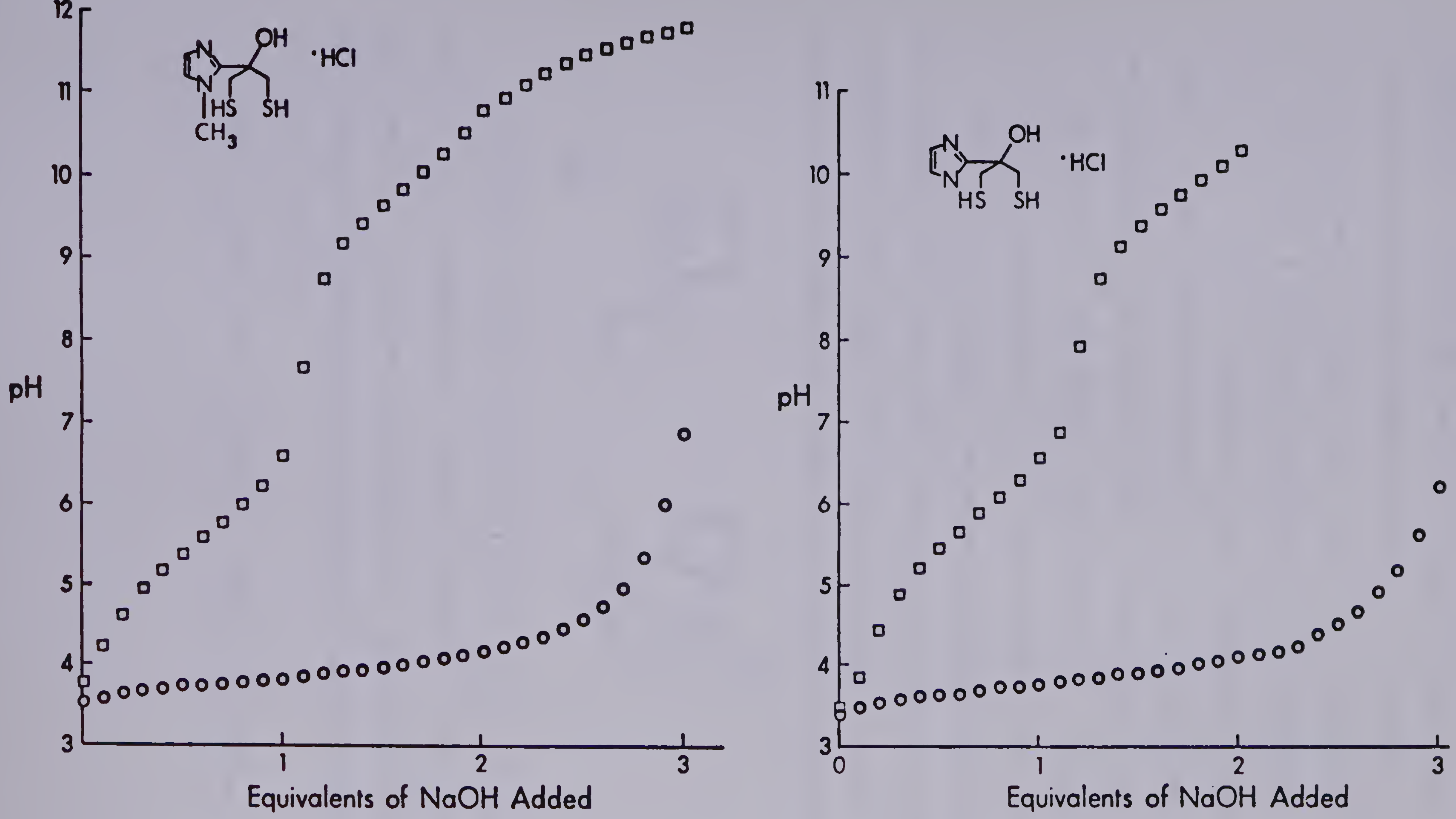
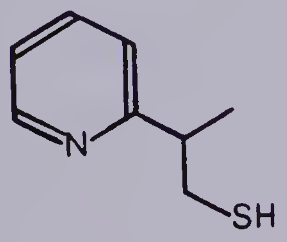
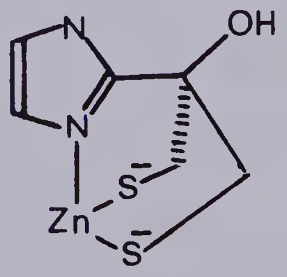


Fig. 5. A plot of the titration curves for imidazoles 38 and 39 (pH vs. equivalents of NaOH added) in the absence (□) and presence (○) of one eq. of Zn^{++} in 50% aq. dioxane.

imidazoles 38 and 39 proved more soluble and their titration curves are shown in Fig. 5. For both species, in the absence of metal, one sees the imidazolium pK_a at ≈ 5.4 and a further titration of a thiol with pK_a ≈ 9.5. In the presence of Zn^{++} however, the situation is again very different; in both cases one sees the consumption of 3 eq. of OH^- at pH values less than 6. On the basis of the above, the additional consumption of OH^- in the presence of Zn^{++} over that in the absence of Zn^{++} (for 38 and 39) can be most adequately explained as having arisen from the formation of stable thiolate complexes such as 50 which may neutralize the metal's charge sufficiently to render the complex insoluble.



49



50

Although visible precipitation occurs with more than 2 eq. of added OH^-, the Co^{++} and Ni^{++} complexes of 38 seem to follow the same behavior, though in both cases the slope of the pH curve is greater than for the zinc case (see Table 2).

The pK_a data for the thiol ligands are shown in Table 3.

TABLE 2. Titration of 38. HCl in the presence and absence of 1 eq. of metal ion (see text for details).

$Eq[OH^-]$	pH	pH(+Zn)	pH(+Co)	pH(+Ni)
0	3.77	3.52	3.64	3.40
0.2	4.62	3.63	3.98	3.60
0.4	5.18	3.69	4.25	3.74
0.6	5.60	3.73	4.37	3.83
0.8	5.99	3.78	4.43	3.90
1.0	6.59	3.83	4.47	3.98
1.2	8.75	3.88	4.53	4.06
1.4	9.42	3.93	4.58	4.17
1.6	9.84	4.00	4.65	4.27
1.8	10.26	4.08	4.74	4.42
2.0	10.77	4.17	4.89	4.62
2.2		4.28	ppt	ppt
2.4		4.45		
2.6		4.74		
2.8		5.37		
3.0		6.88		

TABLE 3. pK_a's for thiol ligands.

Compound	$pK_1^{a,b}$	$pK_2^{a,c}$
31	3.55	10.00
38	5.38	9.61
39	5.45	9.37
47	3.66	10.71
49	4.21	≈10

a. Precision ±0.01 unit. Reported values are the means of 3 determinations.

b. pK_1 refers to $HL^+ \rightleftarrows H^+ + L$

c. pK_2 refers to $L\text{-}SH \rightleftarrows LS^- + H^+$

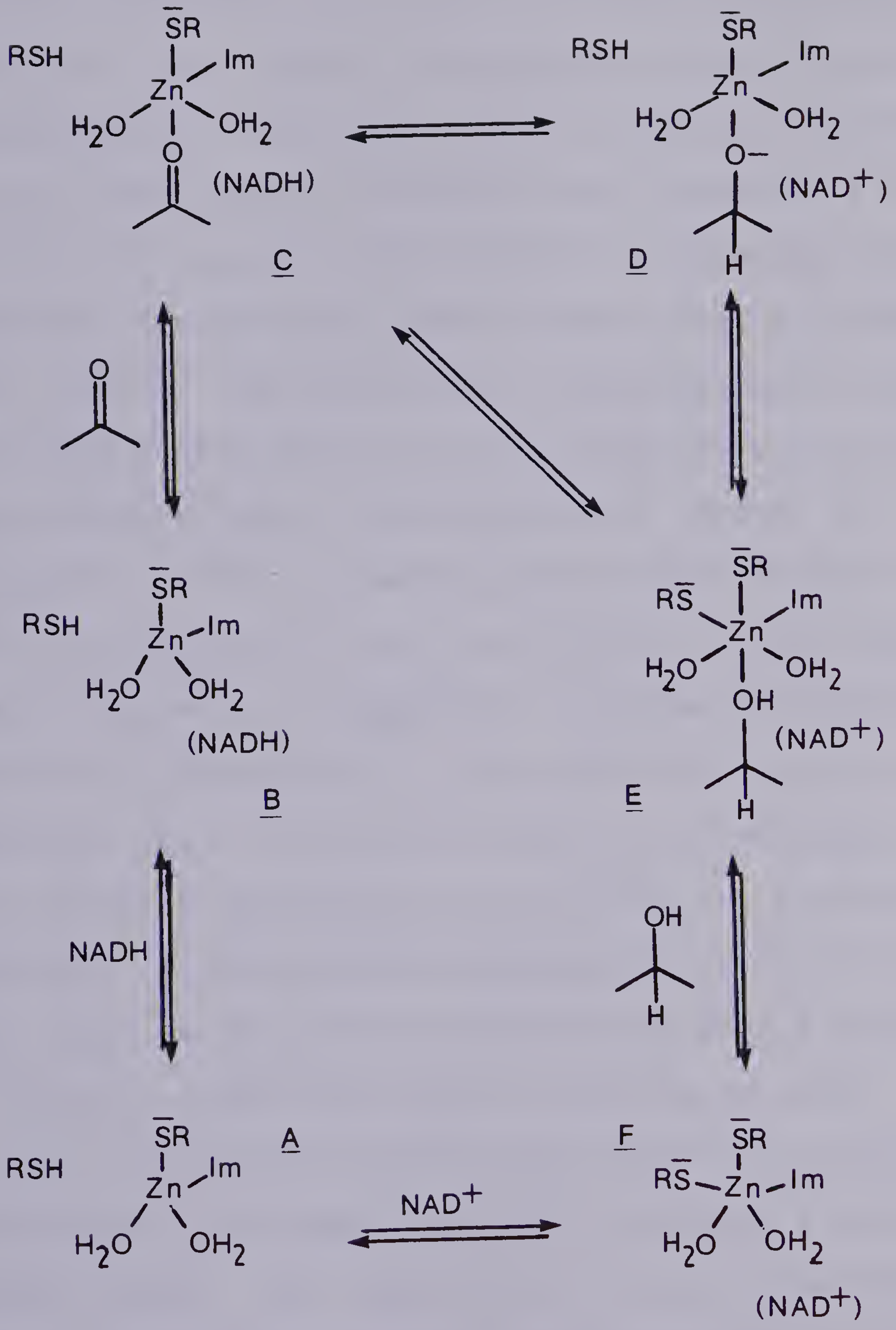
As discussed before, it is expected that inside the active site of the enzyme, the net positive charge of the metal at the time of carbonyl reduction should be an important factor if a Lewis acid – Lewis base interaction is required. The titration data on the metal complexes of ligands 38 and 39 however, indicate that at pH values in excess of 6, (where the enzyme operates) both thiols are completely deprotonated and therefore the Zn^{++} charge is effectively neutralized in these systems. A hydrophobic environment would only serve to favor this charge neutralization.

Whilst on the basis of the above results, one can only speculate about the analogous process in the

enzyme, one might wonder about the Zn^{++} electrophilicity if both the cysteine thiolates are coordinated to the metal during the reduction step. Similarly, one might wonder about mechanisms which involve a zinc bound water ionization if the Zn^{++} already has two anionic groups attached to it. It would seem that if the reason for lowering the pK_a of Zn^{++} bound water relies upon the positive character of the metal stabilizing a bound hydroxide, that reduction of the net positive charge on the metal by ligation to two thiolate groups should seriously reduce the acidity of this bound water.

Consideration of the ionization behavior of the thiol groups on zinc allows speculation about a novel mechanism for enzyme action (Scheme 5) which is consistent with the present and previous solution studies and circumvents the reduced Lewis acid character of $(RS^-)_2Zn^{++}$. In this scheme, the apoenzyme, A, contains a Zn^{++} bound by imidazole and only one thiol (the other two ligands, assuming four coordinate zinc, being water) whilst the second thiol is situated nearby and is protonated. Stepwise addition of NADH and ketone gives C, in which the carbonyl is coordinated to a zinc ion which has some Lewis acid character, as proposed by Dunn.[31] After redox reaction the alcoholate complex, D, is formed and the Zn^{++} is effectively neutralized. Coordination of the pendant thiol, accom-

Scheme 5


RSH
$\bar{S}R$
Im
Zn
H_2O
OH_2
O
(NADH)
C
RSH
$\bar{S}R$
Im
Zn
H_2O
OH_2
O−
(NAD^+)
H
D
NADH
B
$R\bar{S}$
OH
(NAD^+)
E
H
OH
H
A
NAD^+
F

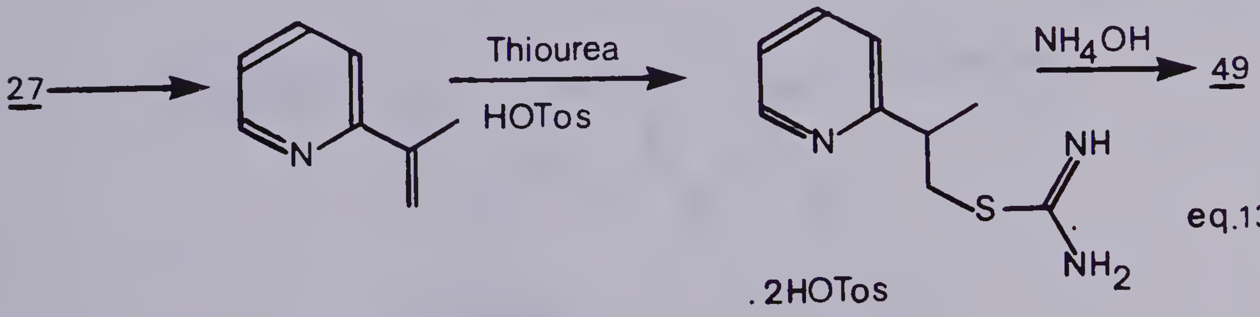
panied by concomitant proton transfer to the liberated alkoxide gives the alcohol complex E. An extra equilibrium has been drawn between C and E to allow for the possibility of concerted proton and hydride transfer. The scheme is consistent with the known observations for the oxidation of alcohols. Coordination of NAD^+ causes a conformational change as a result of which the pendant thiol becomes coordinated and therefore deprotonated, hence proton release accompanies NAD^+ binding. (In this scheme, conformational changes caused by NADH binding is not considered to have any significant effect). Coordination of alcohol to "neutral" zinc is followed by hydride and proton transfer, and debinding of the second thiol to give the ketone species C. Klinman[38] has proposed that the redox reaction is controlled by the action of a single ionizing group ($pK_a = 8.25$) which must be protonated for the reduction reaction and ionized for the oxidation reaction. In the proposed mechanism (Scheme 5), this "pK_a" must be considered to be derived from a combination of factors: the ionization of the thiol and the alcohol, the hydride transfer and the effect of coordinating the thiol(ate), possibly including a conformational change. This "pK_a" is not a pK_a as such but rather a pH below which the reduction reaction is favored and above which the oxidation is favored.

Ligands containing a pyridine and one thiol.

i. Synthesis

As was demonstrated in the last section, the models containing two thiols were found to be unsuitable because of precipitation problems which are believed to be a consequence of the low pH ionization of bound thiol groups. Similarly for ketone 44, we believe the lack of observed reduction may relate to the fact that the Zn^{++}-dithiolate in this system is too poor a Lewis acid to promote carbonyl polarization.

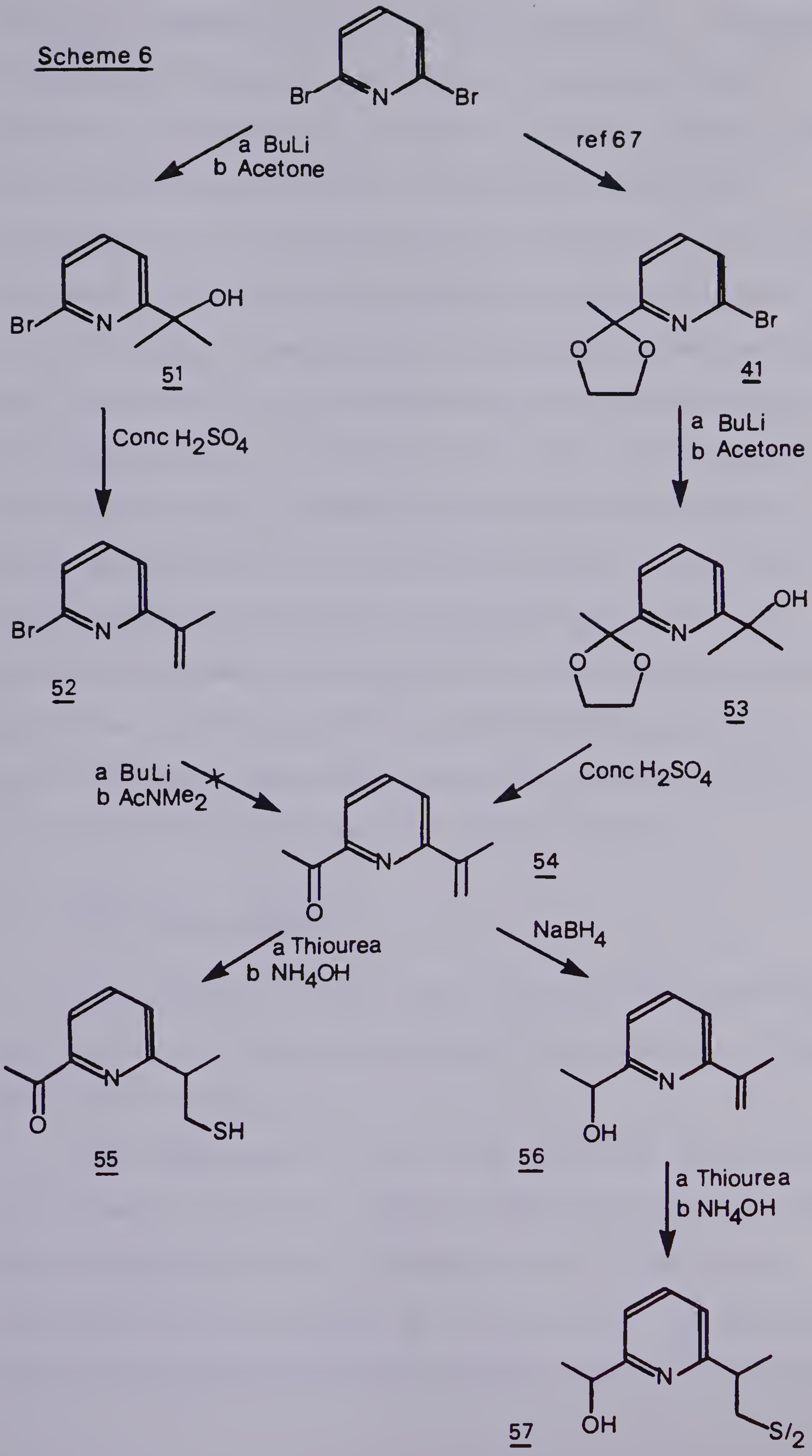
Compound 49 (vide supra) was prepared in the manner described in eq. 13. Dehydration of 27 in hot conc. H_2SO_4 gives the alkene[80] and addition of thiourea across the double bond, the thiouronium salt[81] which is hydrolyzed in ammonia solution to give the thiol 49.[81]



eq.13

The two approaches considered for the synthesis of the 2,6-disubstituted pyridine 55 are shown in Scheme 6. The first attempted route (via 51) involved the reaction of 2-lithio-6-bromopyridine[67] with acetone which gave the adduct in relatively high yield (78%)

Scheme 6


Br
N
Br
a BuLi
b Acetone
ref 67
Br
N
OH
51
O
O
N
Br
41
Conc H_2SO_4
a BuLi
b Acetone
Br
N
52
O
O
N
OH
53
a BuLi
b $AcNMe_2$
Conc H_2SO_4
N
O
54
a Thiourea
b NH_4OH
$NaBH_4$
N
O
SH
55
N
OH
56
a Thiourea
b NH_4OH
57
N
OH
S/2

which was dehydrated to give the alkene 52. Attempted lithiation of 52 gave a mixture of unidentifiable products, possibly as a result of anionic polymerization. The second route (via 41) was however successful. Treatment of the lithium anion of 41 with acetone gave the adduct 53 which was deprotected and dehydrated in conc. H_2SO_4. Quenching of a partially reacted mixture revealed that the deprotection occurred before the dehydration. The thiol derivative was prepared as described for 49. Attempted preparation of the reduced equivalent of 55 (via borohydride reduction of 54 and treatment with thiourea and base) gave the disulphide instead. Although this disulphide is apparently reducible by Zn/CH_3COOH,[82] as evidenced by loss of color, the resultant thiol was very susceptible to air oxidation, rapidly returning to disulphide.

ii. Reduction studies

The reduction of the zinc complex of 55 by BNAH was studied by NMR in CD_3CN/D_2O (7/3) solution using 10^{-2}-10^{-1} M solutions.

Upon addition of 1 eq. zinc chloride, the pH of an equimolar mixture of 55 and BNAH fell rapidly from 10 to below 3 and then increased to ≈ 5 (by glass electrode) over a period of a few hours. Inspection of the NMR spectrum during this period showed the loss of

the CH_3CO singlet at δ 2.70 and the growth of a doublet at δ 2.61. At the same time, the $\underline{CH_3}$-CH doublet at δ 1.3 became more complex with another doublet appearing in almost the same place. The BNAH also disappeared and was replaced by the oxidized NAD^+ analogue (by comparison of the NMR spectrum of authentic material). No evidence of reduction was seen (by comparison with the NMR of 56 and the product of the in situ reduction of 55 by $NaBH_4$). Extraction of the reaction mixture led to the isolation of the species with NMR peaks at δ 2.61 which were thought to arise from the disulphide derived from 55 on the basis of spectral measurements (see exptl).

These observations point to an oxidation of the thiol by molecular oxygen which is probably assisted by the metal coordination.[83] Such a process could lead to a free radical[83] mediated oxidation of the NADH analogue, via a disproportionation process.[84]

In a similar experiment, using 49, the same observations were noted and the disulphide identified in the reaction mixture.

To counter the effect of molecular oxygen, the reaction was studied under anaerobic conditions. Two solutions, one of thiol and one containing BNAH and $ZnCl_2$ were degassed by 10 freeze-thaw cycles. The efficacy of the technique was shown by the fact that only a very small amount of the disulphide of 55 was

observed during NMR monitoring of the attempted reduction.

The reaction observed was extremely complex and whereas the observations can be reported, no explanation of the reactions taking place can be given at this time.

The original CH_3CO peak (δ 2.7) decreased steadily in size with time and a close doublet at δ 2.5 (which may be ascribable to another CH_3CO species) appeared transiently, both sets of signals disappearing in a few hours. At the same time, the BNAH disappeared and was replaced by a lesser amount of the NAD^+ analogue. The methyl region, which showed the appearance of another doublet maintained a constant integration even when the acetyl peaks had disappeared.

Extraction of the reaction mixture gave a residue whose NMR spectrum was similar (minus the peaks due to the NAD^+ analogue) to the original mixture. Upon chromatographic separation of this mixture the major product isolated was the disulphide of 55. Since this species was not present before chromatography then it must have been produced by some process during chromatographic separation. In one case, the reduction product, 57 was identified by NMR, however this result could not be reproduced. Again, this compound was not visible in the extracted mixture.

That 55 reacted with BNAH was shown by two experiments: in the absence of BNAH, no change in the NMR was seen after 1 h. In the presence of 5 eq. of BNAH the reaction proceeded much faster than when 1 eq. was present.

The reaction procedure was repeated with non-reducible $C_6H_5CH_2SH$ instead of 55 and again a reaction was seen in which the NADH analogue disappeared to be replaced by a smaller amount of the oxidized form (NAD^+). In common with the spectra from the preceding cases, many signals were apparent, suggesting formation of a complex reaction mixture.

The above work shows the incompatibility of the zinc complexes of thiols with BNAH under the conditions studied. This would not seem to be a problem for the enzyme since X-ray evidence indicates that the NADH molecule binds some distance away from the ligating cysteine thiols (Fig. 1). Further modelling attempts would require synthesis of a system which is geometrically more similar to that in the enzyme.

Due to the intractable nature of the above reaction mixtures and an inability to observe reduction of the ketone group of 55, further studies with this system seemed fruitless.

EXPERIMENTAL

Synthesis

Routine IR, ^{1}H-NMR, ^{13}C-NMR and mass spectra were recorded on a 7199-Nicolet FT-IR spectrophotometer, a Varian 56/60 (60 MHz) or HA-100 (100 MHz), a Bruker HFX-90 (22.8 MHz) and an AEI MS-50 spectrometer. For column chromatography, silica gel (Merck, 70-230 mesh ASTM) and alumina (Camag, grade III, containing 6% water) were used.

1,3-Di(methylthio)propan-2-ol (23, R = CH_3 X = OH)

This was prepared by the method of Corey[85] et al. in 20% yield, bp 74° (0.1 torr), lit.[85] 110° (7 torr).[86,87]

1,3-Di(phenylthio)propan-2-ol (23, R = C_6H_5 X = OH)[88]

To an ice-cooled solution of 11.5 g (0.5 mol) of Na dissolved in 200 mL abs. EtOH was added 55 g (0.5 mol) of thiophenol over 15 minutes followed by 23.1 g (0.25 mol) of epichlorohydrin over 10 minutes. The temperature during the additions was kept at 50-60° by an ice-water bath. After stirring for 1 h 100 mL of water was added and the mixture extracted with ether (3 x 200 mL). The combined ether layers were dried (Na_2SO_4) and solvent removed in vacuo. The oily residue was purified by column chromatography (250 g

silica, 15 cm column height), eluting with $CHCl_3$, to give 51.4 g (79%) of the product as a colourless oil after removal of solvent: IR 685, 730, 3440 (br) cm^{-1}; NMR (60MHz,$COCl_3$) δ2.9-3.5 (m,5H), 3.6-4.0 (m,1H), 7.0-7.4 (m,10H).[86]

1,3-(Dimethylthio)propan-2-one 24a

Methanethiol (9.2 g (0.2 mol) of liquid (condensed in a cold bath)) was added to a -20° solution of 8.0 g (0.2 mol) NaOH in 100 mL 95% EtOH and the mixture warmed to room temperature. The mixture was then added, over 30 min., to a solution of 12.7 g (0.1 mol) of 1,3-dichloropropan-2-one in 100 mL 95% EtOH with continuous mechanical stirring. After stirring overnight the mixture was poured onto 100 g crushed ice and left 30 min before being extracted with CH_2Cl_2 (3 x 100 mL). The combined CH_2Cl_2 extracts were dried (Na_2SO_4) and solvent removed in vacuo. Fractional distillation of the residue gave 13.0 g (87%) of the product as a pale yellow oil: bp 53° (0.55 torr) lit.[79] 106-9° (9 torr); IR (film) 1706 cm^{-1}; NMR (60MHz,$CDCl_3$) δ2.11 (s,6H), 3.42 (s,4H); mass spectrum m/e (rel. intensity) 150 (M^+, 43), 103 (56), 61 (100); exact mass. calcd. for $C_5H_{10}OS_2$ 150.0173, found 150.0168.

1,3-Di(ethylthio)propan-2-one 24b

This was prepared analogously to 24a except that the reaction was quenched after 4 h and extracted with $CHCl_3$. The product was obtained as 12.9 g (73%) of a pale yellow oil: bp 58° (0.14 torr), lit.[79] 123-4° (11 torr); IR (film) 1250, 1704, 2920, 2960 cm^{-1}; NMR (60MHz,$CDCl_3$) δ1.24 (t,6H), 2.55 (q,4H), 3.45 (s,4H); mass spectrum m/e (rel intensity) 178 (M^+,36), 75 (100); exact mass calcd. for $C_7H_{14}OS_2$ 178.0486, found 178.0491.

1,3-Di(4-N,N-dimethylaminophenylthio)propan-2-one 24c

A solution of 8.0 g (0.2 mol) NaOH in 150 mL abs. ethanol was added over 30 minutes to a mechanically stirred solution of 30.6 g (0.2 mol) of 4-N,N-dimethylaminobenzenethiol and 12.7 g (0.1 mol) 1,3-dichloropropan-2-one in 100 mL abs. ethanol. After stirring overnight, the reaction mixture was poured onto 100 g of crushed ice, with stirring, and left for 30 min. The resultant solid was recrystallized twice from methanol to give 15.5 g (43%) of the product as pale yellow plates: mp 61-2°; IR (cast) 800, 1350, 1500, 1600, 1700 cm^{-1}; NMR (60MHz,$CDCl_3$) δ2.92 (s,12H), 3.64 (s,4H), 6.5-7.4 (m,8H); mass spectrum m/e (rel intensity) 360 (M^+,76), 152 (100); exact mass calcd. for $C_{19}H_{24}N_2OS_2$ 360.1330, found 360.1330.

Anal. calcd. for $C_{19}H_{24}N_2OS_2$: C,63.30; H,6.71; N,7.78; S,17.79. Found: C,63.29; H,6.66; N,7.81; S,17.90.

1,3-Di(4-methoxyphenylthio)propan-2-one 24d

This was prepared as for 24c but in 95% ethanol. On pouring the reaction mixture onto ice an oil was formed which was removed by decanting and induced to crystallize by triturating at -30° in 100 mL methanol. The product was recrystallized from methanol yielding 10.3 g (38%) of white plates: mp 38°; IR (cast) 820, 1240, 1280, 1490, 1706 cm^{-1}; NMR (60MHz,$CDCl_3$) δ3.70 (s),3.78 (s), (together 10H), 6.7-7.5 (m,8H); mass spectrum m/e (rel intensity) 334 (M^+,100), 195 (86), 153 (71); exact mass calcd. for $C_{17}H_{18}O_3S_2$ 334.0697, found 334.0702

Anal. calcd. for $C_{17}H_{18}O_3S_2$: C,61.05; H,5.42; S,19.17. Found: C,61.14; H,5.32; S,19.03.

1,3-Di(4-methylphenylthio)propan-2-one 24e

Prepared analogously to 24c as 20.6 g (68%) of white needles: mp 65-6°, lit.[89] 66-7°; IR (cast) 810, 1240, 1400, 1706 cm^{-1}; NMR (60MHz,$CDCl_3$) δ2.26 (s,6H), 3.73 (s,4H), 6.9-7.3 (m,8H); mass spectrum m/e (rel intensity) 302 (M^+,58), 179 (78), 137 (100); exact mass calcd. for $C_{17}H_{18}O_2$ 302.0799, found 302.07991 .

1,3-Di(phenylthio)propan-2-one 24f

Prepared as for 24c but in 95% ethanol. On pouring the reaction mixture onto ice an oil was formed which was separated by decanting. Two recrystallizations from methanol gave 17.8 g (58%) of the product as pale yellow crystals: mp 36-7°, lit.[70] 42°C; IR (cast) 690, 1440, 1490, 1705 cm^{-1}; NMR (60MHz,$CDCl_3$) δ3.82 (s,4H), 7.3 (m,10H); mass spectrum m/e (rel intensity) 274 (M^+,57), 165 (75), 123 (100); exact mass calcd. for $C_{15}H_{14}OS_2$ 274.0486, found 274.0482.

1,3-Di(4-chlorophenylthio)propan-2-one 24g

Prepared as for 24c but in 95% ethanol (50 mL extra solvent had to be added to the reaction mixture since it solidified), as 22.8 g (67%) of pale yellow needles: mp 78-9°, lit.[90] 89-91°; IR (cast) 800, 810, 1100, 1480, 1712 cm^{-1}; NMR (60MHz,$CDCl_3$) δ3.80 (s,4H), 7.2 (s,8H); mass spectrum m/e (rel intensity) 342 (M^+,56), 156 (100); exact mass calcd. for $C_{15}H_{12}OS_2{}^{35}Cl_2$ 341.9706, found 341.9708.

1,3-Di(3-trifluoromethylphenylthio)propan-2-one 24h

Prepared as for 24c as 24.1 g(59%) of white needles: mp 50-1°; IR (cast) 1130, 1320, 1725 cm^{-1}; NMR (60MHz, $CDCl_3$) δ3.90 (s,4H), 7.4-7.7 (m,8H); mass spectrum m/e

(rel intensity) 410 (M^+,90), 233 (100); exact mass calcd. for $C_{17}H_{12}OF_6S_2$ 410.0234, found 410.0241.

Anal. calcd. for $C_{17}H_{12}OF_6S_2$: C,49.75; H,2.95. Found: C,49.62; H,2.96.

3,5-Dithia-4,4-dimethylcyclohexanol 26

1,3-Dimercaptopropan-2-ol 24.8 g (0.2 mol) was mixed with 11.6 g (0.2 mol) of dry acetone in 200 mL $CHCl_3$ and cooled in ice. To this solution 10 mL of BF_3:etherate was added cautiously and the resultant opaque solution left at 5° for 18 h. This solution was then washed with 100 mL of water and then 100 mL of 0.5 M Na_2CO_3 soln. The $CHCl_3$ layer was dried (Na_2SO_4) and after solvent removal in vacuo was fractionally distilled to give 19.5 g (59%) of the product as a colorless oil. (There is considerable decomposition during distillation; NMR analysis of the reaction mixture indicates a crude yield of >90%): bp 58° (0.08 torr); IR (film) 1040, 2920, 3420 (br) cm^{-1}; NMR (100MHz,$CDCl_3$) δ1.62 (s,3H), 1.74 (s,3H), 2.75 (dd J=14 and 2Hz,2H), 315 (dd J=14 and 5Hz,2H), 3.6 (d,1H,ex), 3.9 (m,1H); mass spectrum m/e (rel intensity) 164 (M^+,83), 74 (100); exact mass calcd. for $C_6H_{12}OS_2$ 164.0329, found 164.0328.

Anal. calcd. for $C_6H_{12}OS_2$: C,43.87; H,7.36; S,39.03. Found: C,43.61; H,7.38; S,38.86.

3,5-Dithia-4,4-dimethylcyclohexanone 24i

This was prepared from 26 in low and variable yields by Moffatt and Lead (IV) acetate/pyridine oxidations: bp 51° (0.2 torr), lit.[71] 57-59° (0.5 torr); NMR (60MHz,$CDCl_3$) δ1.78 (S,6H), 3.46 (s,4H); mass spectrum m/e (rel intensity) 162 (M^+,92), 74 (100), exact mass calcd. for $C_6H_{10}OS_2$ 162.0173, found 162.0169.

2-Lithiopyridine

A solution of 14.2 g (0.09 mol) of 2-bromopyridine in 150 mL dry THF or ether was cooled to -65° under N_2 and 0.09 mol of n-BuLi (≈ 2 M in hexane) was added via syringe such that the temperature did not rise above -55°. After stirring for 15 min. at -50° (not above -50° to avoid decomposition) the red-brown solution was cooled to -65° for subsequent use.

1,3-Dimethylthio-2(2-pyridyl)propan-2-ol 25a

A solution of 4.50 g (0.03 mol) 24a in 50 mL dry THF was added dropwise to 0.09 mol of 2-lithiopyridine (prepared as above) in THF at -65° to -60° over 15 min. After stirring for 2 h at -65° and having warmed to -40°, the mixture was quenched by the addition of 50 mL water and then brought to room temperature. An additional 50 mL water was added and the THF layer separated. The remaining aq. layer was extracted with

ether (2 x 100 mL) and the combined organic layers dried (Na_2SO_4), stripped and the residue put on a vacuum line to remove pyridine. This residue was dissolved in 200 mL ether and dry HCl gas passed into the solution until no more precipitate appeared. The oily precipitate was separated by decanting and crystallized by trituration in ca. 20 mL boiling acetone. Two recrystallizations from $CHCl_3$/methanol yielded 3.24 g (41%) of the HCl salt of 25a as pale brown plates: mp 170-2°; IR (KBr disc) 610, 780, 1070, 1230, 1450, 1520, 1600, 3000, 3250 (br) cm^{-1}; NMR[91] (100MHz,$CDCl_3$) δ2.02 (s,6H), 3.19 (ABq,4H), 4.7 (s,1H,ex), 7.1-7.8 (m,3H), 8.6 (m,1H); mass spectrum m/e (rel intensity) 229 (M^+-HCl,9), 168 (100); exact mass calcd for $C_{10}H_{15}NOS_2$ (M^+-HCl) 229.0595, found 229.0587.

Anal. calcd. for $C_{10}H_{16}ClNOS_2$: C,45.18; H,6.07; N,5.27. Found: C,45.08; H,6.12; N,5.35.

Adduct 25b

This was prepared analogously to 25a and isolated as 3.70 g (42%) of pale brown plates of the HCl salt after two recrystallizations from $CHCl_3$: mp 152-4°; IR (KBr disc) 1080, 1450, 1600, 3000 (br) cm^{-1}; NMR[91] (100MHz,$CDCl_3$) δ1.14 (t,6H), 2.36 (q,4H), 3.15 (ABq,4H), 4.6 (s,1H), 7.1-7.8 (m,3H), 8.6 (m,1H); mass spectrum

m/e (rel intensity) 257 (M^+-HCl,14), 182 (100); exact mass calcd. for $C_{12}H_{19}NOS_2$ (M^+-HCl) 257.0908, found 257.0907.

Anal. calcd. for $C_{12}H_{20}ClNOS_2$: C,49.04; H,6.86; N,4.77. Found: C,48.81; H,6.86; N,4.75.

Adduct 25d

This was prepared analogously to 25a and isolated as 4.65 g (34%) of an off-white powder of the HCl salt after two recrystallizations from $CHCl_3$/methanol: mp 184-6°; IR (KBr disc) 1240, 1490, 3440 (br) cm^{-1}; NMR[91] (100MHz,$CDCl_3$) δ3.46 (ABq,4H), 3.72 (s,6H), 4.8 (s,1H), 6.6-7.6 (m,11H), 8.4 (m,1H); mass spectrum m/e (rel intensity) 274 (M^+-C_7H_7OS,11), 260 (57), 152 (100); exact mass calcd. for $C_{15}H_{16}NO_2S$ (M^+-C_7H_7OS) 274.0902, found 274.0904.

Anal. calcd. for $C_{22}H_{24}ClNO_3S_2$: C,58.72; H,5.38; N,3.11. Found: C,58.65; H,5.17; N,3.08.

Adduct 25e

This was prepared analogously to 25a and isolated as 3.79 g (30%) of cream plates of the HCl salt after two recrystallizations from acetone/ethanol: mp 165-7°; IR (KBr disc) 490, 770, 810, 1490, 3000, 3200, 3440 (br) cm^{-1}; NMR[91] (100MHz,$CDCl_3$) δ2.22 (s,6H), 3.51 (ABq,4H), 4.8 (s,1H), 6.9-7.6 (m,11H), 8.4 (m,1H); mass spectrum

(rel intensity) 381 (M^+-HCl,9), 244 (100); exact mass calcd. for $C_{22}H_{23}NOS_2$ (M^+-HCl) 381.1221, found 381.1220.

Anal. calcd. for $C_{22}H_{24}ClNOS_2$: C,63.21; H,5.79; N,3.35. Found: C,63.23; H,5.59; N,3.35.

Adduct 25f

This was prepared analogously to 25a and isolated as 2.10 g (18%) of pale brown prisms of the HCl salt after two recrystallizations from $CHCl_3$/benzene: mp 120-2°; IR (KBr disc) 690, 740, 1600, 3400 (br) cm^{-1}; NMR[91] (100MHz,$CDCl_3$) δ3.59 (ABq,4H), 4.9 (s,1H), 7.0-7.6 (m,13H), 8.4 (m,1H); mass spectrum m/e (rel intensity) 353 (M^+-HCl,9), 230 (100); exact mass calcd. for $C_{20}H_{19}NSO_2$ (M^+-HCl) 353.0908, found 353.0905.

Anal. calcd. for $C_{20}H_{20}ClNOS_2$: C,61.60; H,5.17; N,3.59. Found: C,61.82; H,5.07; N,3.56.

Adduct 25g

This was prepared analogously to 25a and isolated as 2.79 g (20%) of cream crystals of the HCl salt after two recrystallizations from $CHCl_3$/methanol: mp 175-8°; IR (KBr disc) 490, 820, 1090, 1480, 3440 (br) cm^{-1}; NMR[91] (100MHz,$CDCl_3$) δ3.51 (ABq,4H), 4.9 (s,1H), 6.9-7.6 (m,11H), 8.4 (M,1H); mass spectrum m/e (rel intensity) 421 (M^+-HCl,5), 264 (100); exact mass calcd. for $C_{20}H_{17}{}^{35}Cl_2NOS_2$ 421.0128, found 421.0118.

Anal. calcd. for $C_{20}H_{18}Cl_3NOS_2$: C,52.35; H,3.95; N,3.05. Found: C,52.30; H,4.00; N,3.09.

Adduct 25h

This was prepared analogously to 25a and isolated as 2.17 g (14%) of white crystals of the HCl salt after two recrystallizations from $CHCl_3$/ether: mp 139-40°; IR (KBr disc) 1120, 1320, 3440 (br) cm^{-1}; NMR[91] (100MHz, $CDCl_3$) δ3.57 (ABq,4H), 5.0 (s,1H), 6.9-7.6 (m,11H), 8.4 (m,1H); mass spectrum m/e (rel intensity) 489 (M^+-HCl,4), 298 (100); exact mass calcd. for $C_{22}H_{17}NOS_2F_6$ 489.0657, found 489.0657.

Anal. calcd. for $C_{22}H_{18}ClF_6NOS_2$: C,50.24; H,3.45; N,2.66. Found: C,50.05; H,3.38; N,2.62.

Adduct 25i

This was prepared analogously to 25a but was isolated according to the following procedure. The crude product (after removal of pyridine) was dissolved in 200 mL ether and extracted with 0.1 M HCl (3 x 100 mL). The combined acid extracts were neutralized with solid $NaHCO_3$ and extracted with $CHCl_3$ (3 x 100 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and solvent removed in vacuo. The product was purified by column chromatography (100 g silica gel, 40 cm column height), eluting with $CHCl_3$, after removal of solvent

1.64 g (23%) of pale yellow needles were obtained: mp 46-7°; IR (cast) 750, 1060, 1440, 1590, 3440 (br) cm^{-1}; NMR (100MHz,$CDCl_3$) δ1.64 (s,3H), 1.93 (s,3H), 2.50 (d,J=14Hz,2H), 3.86 (d,J=14Hz,2H), 4.5 (s,1H), 7.1-7.9 (m,3H), 8.5 (m,1H); mass spectrum m/e (rel intensity) 241 (M^+,61), 223 (84), 190 (64), 121 (100; exact mass calcd. for $C_{11}H_{15}NOS_2$ 241.0595, found 241.0593.

Anal. calcd. for $C_{11}H_{15}NOS_2$: C,54.74; H,6.26; N, 5.83; S,26.57. Found: C,54.86; H,6.37; N,5.57; S, 26.85.

2-(2-Pyridyl)-propan-2-ol 27

This was prepared as previously described[73] in 22% yield as white needles: mp 48-9°, lit.[73] 49-50°; IR (cast) 790, 1180, 2950, 3400 (br) cm^{-1}; NMR (60MHz,$CDCl_3$) δ1.58 (s,6H), 5.1 (s,1H), 7.0-7.8 (m,3H), 8.5 (m,1H); mass spectrum m/e (rel intensity) 122 (M^+-CH_3,100); exact mass calcd. for C_7H_8NO (M^+-CH_3) 122.0606, found 122.0606.

This compound could also be made from 2-lithiopyridine and acetone (1:1 ratio) in 50% yield after recrystallization from Skelly B. (A purer product is obtained by distillation).

1,3-Dimercaptoacetone

This was prepared as described previously[78] in >80% yield: mp 105-9° (in another case 102-4°), lit[78]

85-6°: IR (KBr disc) 1110, 2550 (w), 3450 (br) cm^{-1}; NMR (60MHz,d_6-DMSO) δ2.0-2.4 (dd,2H), 2.7-3.5 (m,8H), 5.9 (s,2H); mass spectrum m/e (rel intensity) 225 (M^+-H_2O,38), 122 (100); exact mass calcd. for $C_3H_6OS_2$ 121.9860, found 121.9863.

1,3-Bis(ethoxymethylthio)propan-2-one 32

12.2 g (0.1 mol) of 1,3-dimercaptoacetone were dissolved in 300 mL of dry THF, under N_2, and the solution cooled to -5°. Two eq. of n-BuLi (≈ 2 M in hexane) was then added such that the temperature stayed in the range -5-0°. After stirring for 1 h, the cream suspension was cooled to -65° and 20.8 g (0.22 mol) of chloromethylethyl ether in 50 mL dry THF was added over 30 min. The mixture was left to warm up and stir for 8 h and 200 mL of water was added. The THF layer was separated and the aq. layer extracted with ether (2 x 100 mL). The combined organic extracts were dried (Na_2SO_4) and solvent removed in vacuo. Distillation of the residue from a pre-heated oil bath (130°) gave 18.8 g (80%) of the product as a pale yellow oil: bp 115° (0.1 torr); IR (film) 1080, 1708 cm^{-1}; NMR (60MHz, $CDCl_3$) δ1.18 (t,6H), 3.55 (s),3.56 (q) (together 8H), 4.65 (s,4H); mass spectrum m/e (rel intensity) 238 (M^+,4), 59 (100); exact mass calcd. for $C_9H_{18}O_3S_2$ 238.0698, found 238.0698.

Anal. calcd. for $C_9H_{18}O_3S_2$: C,45.35; H,7.61; S, 26.90. Found: C,45.36; H,7.67; S,26.88.

1,3-Dimercapto-2-(2-pyridyl)propan-2-ol 31

A solution of 7.14 g (0.03 mol) 32 in 50 mL dry ether was added dropwise over 30 min. to 0.09 mol of 2-lithiopyridine (prepared as above) in 150 mL ether at -65°. After 2 h stirring, the mixture was warmed to -40° and 50 mL sat. NH_4Cl added. After the solution had been brought to >0°C, 200 mL water was added, the ether layer separated and the aq. layer extracted with ether (2 x 100 mL). The combined ether extracts were dried (Na_2SO_4) and the volatiles removed in vacuo. The residue was then dissolved in 200 mL ether and extracted with 3 M HCl (6 x 50 mL). Neutralization of the combined acid extracts with solid K_2CO_3 followed by $CHCl_3$ extraction (4 x 100 mL), drying (Na_2SO_4) and evaporation of solvent gave the addition product in reasonably pure form. NMR (60MHz,$CDCl_3$) δ1.2 (t), 3.3 (ABq), 3.5 (q), 4.6 (s), 5.0 (s,br), 7.0-7.7 (m), 8.4-8.6 (m); exact mass calcd. for $C_{14}H_{23}NO_3S_2$ 317.1120, found 317.1120.

This residue was then dissolved in 20 mL of 4:1 (v/v) CH_3CN/H_2O and a solution of 16.3 g (0.06 mol) $HgCl_2$ in 130 mL of the same solvent was added in one portion. A white precipitate rapidly formed and the mixture became acidic (as judged by indicator paper).

After 4 h stirring, the mixture was filtered and the precipitate washed with 200 mL water. The solid was then slurried with 200 mL water with rapid stirring and H_2S passed through for 6 h after which the resultant black mixture was treated with solid K_2CO_3 to raise the pH to 8 and then continuously extracted with ether, for 48 h, under a N_2 atmosphere. The ether extract was dried (Na_2SO_4) and dry HCl gas passed through. The resultant oil crystallized on standing and was isolated by decanting and washing with ether to give 1.75 g (25%) of the HCl salt of 31 as pale yellow crystals: mp 158-60°; IR (KBr disc) 770, 1080, 1520, 1600, 3000, 3250 (br) cm^{-1}; NMR (100MHz,D_2O) δ3.29 (s,4H), 8.0-8.2 (m,2H), 8.6-8.9 (m,2H) (integration of the HOD peak showed that 4H had been exchanged); mass spectrum m/e (rel intensity) 201 (M^+-HCl,0.4), 168 (39), 154 (100); exact mass calcd. for $C_8H_{10}NOS$ (M^+-HCl-SH) 168.0483, found 168.0486.

Anal. calcd. for $C_8H_{12}ClNOS_2$: C,40.41; H,5.09; N,5.89; O,6.73; Cl,14.91. Found: C,40.54; H,5.14; N, 6.07; O,6.97; Cl,14.87.

Compound 36

4.88 g (0.04 mol) of 1,3-dimercaptoacetone and 3.20 g (0.08 mol) of NaOH were dispersed in 50 mL water. To this solution was added 7.56 g (0.08 mol) of chloro-

methylethyl ether in 5 mL ether over 10 min., an exothermic reaction occurred and after 30 min. stirring, the acidic solution was extracted with ether (2 x 50 mL). The combined ether extracts were dried (Na_2SO_4) and solvent removed in vacuo. The resultant residue was separated by fractional distillation and that portion boiling at 68°C (0.13 torr) was collected. The pot residue was identified as impure 32 by NMR. The distillate was further purified by column chromatography (100 g silica gel, 40 cm column height), eluting with $CHCl_3$ to give 1.40 g (21%) of white plates after removal of solvent: mp 37-9°; IR (cast) 1035 cm^{-1}; NMR (100MHz, $CDCl_3$) δ3.24 (ABq,4H), 4.92 (ABq,4H); ^{13}C NMR (22.6MHz, $CDCl_3$) δ38.2, 69.2 (both triplets in off resonance spectrum); mass spectrum m/e (rel intensity) 164 (M^+, 100), 133 (28), 60 (52); exact mass calcd. for $C_5H_8O_2S_2$ 163.9966, found 163.9961.

Anal. calcd. for $C_5H_8O_2S_2$: C,36.56; H,4.91; O,19.48; S,39.04. Found: C,36.76; H,4.88; O,19.54; S,39.27.

1,3-Dimercapto-2-(N-methyl-2-imidazolyl)propan-2-ol 38

This was obtained by the addition of 0.03 of 32 in 50 mL ether to 0.09 mol of 2-lithio-N-methylimidazole (prepared by the addition of n-BuLi to N-methylimidazole) at -40°C in 150 mL 2:1 (v/v) ether/THF. The work-up and deblocking procedure was as for 31 with the

exception that the final ether extract was dried (Na_2SO_4) and solvent removed in vacuo to give 1.04 g (17%) of the product as a white powder: mp 119-21°; IR (cast) 1060, 1470, 2600 (w), 3060 (br) cm^{-1}; NMR (100MHz,$CDCl_3$,-50°C) δ1.6 (dd,2H), 2.8-3.5 (m,4H), 3.87 (s,3H), 4.9 (s,1H,br), 6.8 (m,2H) (at RT considerable exchange occurs, the thiol protons became a broad singlet, the multiplet around δ3 simplified to a ABq and the OH proton was not discernible); mass spectrum m/e (rel intensity) 187 (M^+-OH,2), 157 (53), 114 (100); exact mass calcd. for $C_7H_{11}N_2S_2$ (M^+-OH) 187.0364, found 187.0364.

Anal. calcd. for $C_7H_{12}N_2OS$: C,41.15; H,5.92; N, 13.71; S,31.39. Found: C,41.28; H,5.90; N,13.70; S,31.55.

1,3-Dimercapto-2(2-imidazolyl)propan-2-ol 39

This was prepared by the addition of 0.03 mol of 32 in 30 mL ether, over 30 min., to a solution of 2-lithio-1-diethoxymethylimidazole (see succeeding section) in 150 mL ether at -40°. The mixture was stirred for 1 h at -60° and allowed to warm to 0° when 20 mL water were added. The ether layer was separated and the aq. layer was extracted with ether (2 x 50 mL). The combined ether layers were dried (Na_2SO_4) and solvent removed in vacuo. The residue was then stirred

in 100 mL 50% aq. ethanol for 4 h, the ethanol removed under reduced pressure and the residue extracted with $CHCl_3$. The $CHCl_3$ solution was extracted with 0.2 M HCl (4 x 200 mL), the combined acid extracts neutralized with solid Na_2CO_3 and extracted with $CHCl_3$ (4 x 100 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and solvent removed in vacuo to give 3.30 g (36%) of the adduct which could be recrystallized from ether/$CHCl_3$ to give white needles: mp 100-1°; IR (cast) 1060, 1090 cm^{-1}; NMR (60MHz,$CDCl_3$) δ1.19 (t,3H), 3.30 (s,4H), 3.56 (q,4H), 4.53 (s,1H), 7.00 (s,2H); mass spectrum m/e (rel intensity) 306 (M^+,0.2), 201 (47), 155 (100); exact mass calcd. for $C_{12}H_{22}N_2O_3S_2$ 306.1072, found 306.1080.

Anal. calcd. for $C_{12}H_{22}N_2O_3S_2$: C,47.03; H,7.24; N,9.14; S,20.93. Found: C,46.75; H,7.22; N,9.15; S, 20.69.

The hemithioketal was then treated as before and the product isolated as for 38 as 1.08 g (20% overall) of pale yellow crystals: mp 106-7°; IR (cast) 1090, 2560 (w), 3300 (br) cm^{-1}; NMR (100MHz,CD_3OD) 3.02 (s,4H), 6.96 (s,2H); mass spectrum m/e (rel intensity) 157 (M^+-SH,17), 143 (100), 69 (84); exact mass calcd. for $C_6H_9N_2OS$ (M^+-SH) 157.0436, found 157.0434.

Anal. calcd. for $C_6H_{10}N_2OS_2$: C,37.87; H,5.30; N, 14.72; O,8.41; S,33.70. Found: C,38.21; H,5.24; N,14.51; O,8.64; S,34.03.

6-Bromo-2-acetylpyridine

Prepared in 87% yield, as described previously:[67a] mp 52-3°, lit.[67a] 54-5°.

6-Bromo-2-(2-methyl-1,3-dioxolan-2-yl)pyridine 41

Prepared in 82% yield as previously described:[67a] mp 42-4°, lit.[67a] 44-6°.

1,3-Dimercapto-(6-acetyl-2-pyridyl)propan-2-ol 44

7.14 g (0.03 mol) of 32 in 50 mL ether was added over 30 min. to a suspension of 0.075 mol the lithium anion of 41,[67a] prepared as for 2-lithiopyridine, in 150 mL ether at -65°C. After 2 h stirring at -65°C the mixture was warmed to -40°C, quenched with 50 mL sat. NH_4Cl solution and warmed to >0°. This mixture was then extracted with 0.1 M HCl (3 x 150 mL) (removes debrominated 41) and then with 3 M HCl (4 x 100 mL). The latter extract, which contains the adduct was left for 24 h to effect deprotection of the ketone. This solution was then neutralized with solid K_2CO_3 and extracted with $CHCl_3$ (3 x 100 mL), the combined $CHCl_3$ extracts were dried (Na_2SO_4) and evaporated under reduced pressure. The residue was cleaved with 0.06 mol $HgCl_2$, as described for 31. The resultant mercury(II) salt was then slurried in 100 mL MK_2CO_3 soln. and H_2S passed

through for 20 min. with the pH being maintained at 9. After a further 10 min. standing the mixture was extracted with $CHCl_3$ (4 x 100 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and the solvent removed in vacuo. The resultant residue was purified by column chromatography (50 g silica gel, 30 cm height), eluting with $CHCl_3$ to give 0.78 g (11%) of the product as a pale yellow viscous oil after evaporation of solvent: IR (cast) 1360, 1690, 2560 (w), 3460 (br) cm^{-1}; NMR (100MHz, $CDCl_3$) δ1.36 (t,2H), 2.70 (s,3H), 2.9-3.3 (m,4H), 4.4 (s,1H), 7.7-8.1 (m,3H); mass spectrum m/e (rel intensity) 210 (M^+-SH,46), 196 (100, 164 (55); exact mass, calcd. for $C_{10}H_{12}NO_2S$ (M^+-SH) 210.0588, found 210.0588.

Anal. calcd. for $C_{10}H_{13}NO_2S_2$: C,49.36; H,5.38; N,5.76; O,13.15. Found: C,49.65; H,5.28; N,5.99; O,13.02.

1,3-Dimercapto-2(6-(1-hydroxyethyl)-2-pyridyl)propan-2-ol 46

4.30 g (0.012 mol) of crude 43 was prepared as described above and treated with 0.91 g (0.024 mol) $NaBH_4$ in 50 mL abs. ethanol for 1 h. Then 20 mL of water was added and the mixture continuously extracted with ether overnight. The ether extract was dried (Na_2SO_4) and solvent removed in vacuo (the NMR spectrum showed the absence of an acetyl group). The residue

was cleaved by the action of 6.52 g (0.024 mol) $HgCl_2$ to give 46 as its HCl salt, isolated as for 31 as 0.74 g (9%) of a white powder: mp 140-2°; IR (KBr disc) 1620, 3200 (br cm^{-1}; NMR (100MHz,D_2O) δ1.85 (d,3H), 3.46 (s,4H), 5.60 (q,1H), 8.1-8.3 m,2H), 8.7-9.0 (m,1H); mass spectrum m/e (rel intensity) 212 (M^+-HCl-SH,65), 198 (91), 180 (100), 166 (59); exact mass calcd. for $C_{10}H_{14}NO_2S$ (M^+-HCl-SH) 212.0745, found 212.0743.

Anal. calcd. for $C_{10}H_{16}ClNO_2S_2$: C,42.62; H,5.72; N,4.97; O,11.35; S,22.75. Found: C,42.65; H,5.60; N,4.78; O,11.63; S,22.70.

1-Mercapto-2-(2-pyridyl)-3-methylthiopropan-2-ol 47

This was prepared by the addition of 4.27 g (0.022 mol) of 48 in 20 mL ether, over 15 min. to 0.066 mol 2-lithiopyridine (made as described before) in 120 mL ether at -65°. The work-up procedure was as for 31 except that 6.0 g (0.022 mol) of $HgCl_2$ was used to effect cleavage and 1.15 g (21%) of the HCl salt of 47 was obtained as a white powder: mp 162-4°; IR (KBr disc) 1460, 1520, 1605, 2580 (w), 3200 (br) cm^{-1}; NMR (100MHz,D_2O) δ2.35 (s,3H), 3.58 (s), 3.61 (s) (together 4H), 8.3-8.6 (m,2H), 8.9-9.2 (m,2H), integration of the HOD peak shows 3 protons exchanged; mass spectrum m/e (rel intensity) 215 (M^+-HCl,3), 168 (53), 154 (100); exact mass calcd. for $C_9H_{13}NOS_2$

215.0438, found 215.0434.

Anal. calcd. for $C_9H_{14}ClNOS_2$: C,42.93; H,5.60; N,5.56; S,25.47. Found: C,42.96; H,5.64; N,5.51; S,25.34.

1-Methylthio-3-ethoxymethylthiopropan-2-one 48

To 12.2 g (0.1 mol) of 1,3-dimercaptoacetone in 300 mL THF at -5° was added 0.1 mol n-BuLi (≈ 2 M in hexane) such that the temperature was ca. -5°. After stirring at -5° for 30 min., the suspension was cooled to -65° and 9.85 mL (0.1 mol) of chloromethylethyl ether added over 5 min. and the cold bath removed. After 15 min. the ppt had dissolved and the mixture cooled to -65° prior to the addition of 0.1 mol of n-BuLi. Then 4.8 mL (0.12 mol) of CH_3I was added to the resultant clear solution over 5 min. and the mixture left to stir and warm up for 8 h. The reaction mixture was then poured on 100 mL water and the THF layer separated and the aq. layer extracted with ether (2 x 100 mL). The combined organic layers were dried (Na_2SO_4) and solvent removed in vacuo. Three fractional distillations gave 4.7 g (24%) of a pale yellow liquid which was free of 24a and 32 by mass spectrometry: bp 75° (0.05 torr); IR (film) 1080, 1704 cm^{-1}; NMR (60MHz,$CDCl_3$) δ1.19 (t,3H), 2.08 (s,3H), 3.37 (s), 3.55 (s), 3.56 (q) (together 6H), 4.66 (s,2H);

mass spectrum m/e (rel intensity) 194 (M^+,25), 104 (43), 59 (100); exact mass calcd. for $C_7H_{14}O_2S_2$ 194.0434, found 194.0434.

Anal. satisfactory analyses could not be obtained for this compound.

2-(2-Pyridyl)propene

This was prepared from 27 as previously described[80] but with a reaction time of 15 min. at 120° and not 3 h (under which conditions little or no product is obtained), in 57% yield: bp 74° (15 torr) lit.[80] 63-7° (10 torr).

2-(2-pyridyl)propan-1-thiol 49

2.70 g (0.0227 mol) of 2-(2-pyridyl)propene, 9.49 g (0.0499 mol) of p-toluenesulphonic acid monohydrate and 1.73 g (0.0227 mol) thiourea were refluxed in 30 mL abs. ethanol for 2 h. After cooling to room temperature, half the solvent was evaporated under reduced pressure and 30 mL of ether added to the residue and the mixture left to crystallize. The ditosylate of the thiouronium salt 6.81 g (56%) was collected as white crystals: mp 167-70°. Anal. calcd. for $C_{23}H_{19}N_3O_6S_3$: C,51.19; H,5.42; N,7.79. Found: C, 51.12; H,5.43; N,7.55.

The salt 6.70 g (0.0125 mol) was refluxed in

50 mL 4:1 conc. NH_3:water for 1 h and the cooled solution extracted with $CHCl_3$ (4 x 100 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and solvent removed in vacuo. Kugelrohr distillation (oven temp. 83°, 0.5 torr) of the residue gave 1.32 g (69%) of 49 as a colourless oil: IR 750, 1430, 1450, 1590, 2550 (w) cm^{-1}; NMR (100MHz,$CDCl_3$) δ1.2-1.5 (m,4H, one of which is exchangeable), 2.6-3.2 (m,4H), 7.0-7.8 (m,3H), 8.6 (m,1H); mass spectrum m/e (rel intensity) 152 (M^+-H, 100), 120 (32); exact mass calcd. for $C_8H_{10}NS$ (M^+-H) 152.0534, found 152.0530.

Anal. calcd. for $C_8H_{11}NS$: C,62.70; H,7.24; N,9.14. Found: C,62.86; H,7.28; N,9.14.

2-(6-Bromo-2-pyridyl)propan-2-ol 51

Acetone (6.38 g (0.11 mol)) was added over 15 min. to a solution of 0.10 mol of 2-lithio-6-bromopyridine in 250 mL dry ether, under N_2, at -65°. After 2 h stirring at -65°, 50 mL of sat. NH_4Cl solution was added and the mixture warmed to >0°. The ether layer was separated and the aq. layer extracted with more ether (2 x 50 mL). The combined ether layers were dried (Na_2SO_4) and solvent removed in vacuo. Purification by column chromatography (200 g silica gel, column height 20 cm), eluting with $CHCl_3$, gave a residue which was distilled to give 16.9 g (78%) of 51 which crys-

tallized as white needles on standing: mp 33-5°, bp 69° (0.25 torr); IR 1560, 3420 (br) cm^{-1}; NMR (60MHz, $CDCl_3$) δ1.55 (s,6H), 4.1 (s,1H,br), 7.2-7.8 (m,3H); mass spectrum m/e (rel intensity) 120 (M^+-CH_4Br,9), 102 (27), 78 (100); exact mass calcd. for C_7H_6NO (M^+-CH_4Br) 120.0449, found 120.0448.

Anal. calcd. for $C_{10}H_{10}BrNO$: C,44.47; H,4.66; N,6.48; O,7.40. Found: C,44.65; H,4.69; N,6.42; O,7.12.

2-(6-Bromo-2-pyridyl)propene 52

4 g (0.0185 mol) of 51 was added to 12 mL conc. H_2SO_4 and the mixture heated at 120° for 15 min. The hot solution was then poured on 30 g ice/water and neutralized with solid K_2CO_3. This mixture was extracted with $CHCl_3$ (4 x 50 mL), the combined $CHCl_3$ extracts dried (Na_2SO_4) and solvent removed under reduced pressure. Kugelrohr distillation (oven temp. 90°, 2.5 torr) gave 2.26 g of 52 as a colorless oil: NMR (60MHz,$CDCl_3$) δ2.2 (m,3H), 5.3 (m,1H), 5.9 (m,1H), 7.2-7.7 (m,3H); mass spectrum m/e (rel intensity) 199 (M^++2,92), 197 (M^+,100), 117 (74); exact mass calcd. for $C_8H_8N^{79}Br$ 196.9840, found 196.9835.

Anal. satisfactory analyses could not be obtained for this compound.

2-((6-Methyl-1,3-dioxolan-2-yl)-2-pyridyl)propan-2-ol 53

Acetone (4.99 mL (0.0681 mol)) was added over 10 min. to a solution of the 2-lithioanion of 41 in 250 mL THF, under N_2, at -65°. After stirring for 3 h at -65°, 50 mL sat. NH_4Cl solution was added and warmed to >0°. Water (50 mL) was added, the THF layer separated and the aq. layer extracted with 50 mL ether. The combined organic layers were dried (Na_2SO_4), solvent removed in vacuo and the residue fractionally distilled. Two fractions were collected, the more volatile (bp 65°, 0.1 torr) was debrominated 41, whilst the latter contained 7.55 g (55%) of 53 isolated as a colorless viscous oil: bp 91° (0.1 torr); IR (film) 1080, 1200, 3400 (br) cm^{-1}; NMR (100MHz,$CDCl_3$) δ1.54 (s,6H), 1.75 (s,3H), 3.8-4.2 (m,4H), 5.2 (s,1H,br), 7.2-7.8 (m,3H); mass spectrum m/e (rel intensity) 208 (M^+-CH_3,11), 87 (100); exact mass calcd for $C_{11}H_{14}NO_3$ (M^+-CH_3) 208.0974, found 208.0977.

Anal. calcd. for $C_{12}H_{17}NO_3$: C,64.55; H,7.67; N, 6.27. Found: C,64.23; H,7.79; N,6.14.

2-(6-Acetyl-2-pyridyl)propene 54

5.0 g (0.0224 mol) of 53 was added to 10 mL conc. H_2SO_4 and the mixture heated at 120° for 10 min. The hot solution was then poured onto a slurry of 50 g

$NaHCO_3$ in 200 mL water and the mixture extracted with $CHCl_3$ (3 x 100 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4), solvent removed under reduced pressure and the residue distilled in a Kugelrohr tube (oven temp. 101°, 0.7 torr) to give 2.82 g (78%) of 54 as a colorless oil: IR (film) 820, 1350, 1580, 1695 cm^{-1}; NMR (100MHz,$CDCl_3$) δ2.3 (m3H), 2.72 (s,3H), 5.35 (m,3H), 5.95 (m,1H), 7.5-8.0 (m,3H); mass spectrum m/e (rel intensity) 161 (M^+,100), 118 (76); exact mass calcd. for $C_{10}H_{11}NO$ 161.0841, found 161.0841.

Anal. calcd. for $C_{10}H_{11}NO$: C,74.51; H,6.88; N,8.69. Found C,74.43; H,7.04; N,8.71.

2-(6-Acetyl-2-pyridyl)propan-1-thiol 55

1.68 g (0.0104 mol) 54 0.79 (0.0104 mol) thiourea and 4.35 g (0.0229 mol) of p-toluenesulfonic acid monohydrate were refluxed in 20 mL abs. ethanol for 2 h. After cooling the mixture was concentrated on a rotary evaporator and the resultant residue refluxed for 2 h in 25 mL of a 4:1 (v/v) mixture of conc. NH_3 and water. The cooled solution was extracted with $CHCl_3$ (6 x 50 mL), the combined $CHCl_3$ extracts dried (Na_2SO_4) and solvent removed in vacuo. The resultant brown oil was purified by column chromatography (50 g silica gel, column height 40 cm), eluting with $CHCl_3$, giving 0.96 g (47%) of 55 as a colorless oil after

removal of solvent in vacuo: IR (film) 1360, 1590, 1690, 2560 (w) cm^{-1}; NMR (100MHz,$CDCl_3$) δ1.2-1.5 (m, 4H, one of which is exchangeable), 2.70 (s) 2.6-3.3 (m) (together 6H), 7.2-7.3 (m,1H), 7.6-7.9 (m,2H); mass spectrum 194 (M^+-H,21), 162 (100); exact mass calcd. for $C_{10}H_{12}NOS$ (M^+-H) 195.0718, found 195.0710.

Anal. calcd. for $C_{10}H_{13}NOS$: C,61.50; H,6.71; N,7.17. Found: C,61.77; H,6.75; N,7.09.

2-(6-(1-hydroxethyl)-2-pyridyl)propene 56

2.50 g (0.0155 mol) of 54 was treated with 0.293 g (0.00775 mol) $NaBH_4$ in 50 mL abs. ethanol for 1 h (TLC analysis showed the reaction was complete). Then 50 mL of water was added and the mixture extracted with $CHCl_3$ (3 x 100 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and solvent removed in vacuo. Kugelrohr distillation (oven temp. 92°, 0.4 torr) gave 2.40 g (95%) of the product as a colorless oil: IR (film) 1450, 1570, 1580, 2970, 3400 cm^{-1}; NMR (100MHz, $CDCl_3$) δ1.48 (d,3H), 2.2 (m,3H), 4.84 (q,1H), 4.7-5.0 (br,1H,ex), 5.3 (m,1H), 5.9 (m,1H), 7.0-7.7 (m,3H); mass spectrum m/e (rel intensity) 163 (M^+,87), 148 (95), 144 (100); exact mass calcd. for $C_{10}H_{13}NO$ 163.0997, found 163.0998.

Anal. satisfactory analysis could not be obtained for this compound.

Attempted preparation of the reduced form of 55

The procedure used was that outlined for 55 starting from 2.20 g (0.0135 mol) of 56, 1.03 g (0.0135 mol) thiourea and 5.64 g (0.0297 mol) of p-toluenesulphonic acid monohydrate. The crude product was purified by column chromatography (100 g grade 3 alumina, column height 35 cm) eluting with $CHCl_3$. After evaporation of solvent in vacuo a viscous yellow oil was obtained which did not discolor iodine. On the basis of this and spectral evidence it was thought that the disulphide 57 had been prepared: NMR (100MHz,$CDCl_3$) δ1.2-1.6 (dd,12H), 2.5-3.2 (m,6H), 4.82 (q,2H), 4.6-5.0 (br, 2H,ex), 6.9-7.1 (m,4H), 7.6 (t,2H) (D_2O exchange had no effect on the two high field signals); mass spectrum m/e (rel intensity) 318 ($M^+-C_3H_6S$,17), 196 ($M^+/2$,55), 164 (100); exact mass calcd. for $C_{10}H_{14}NOS$ ($M^+/2$) 196.0814, found 196.0805.

Potentiometric titrations

i. pK_a determinations

These were performed in a jacketted cell kept at 25 ± 0.1°. Air was excluded from the cell by passing a gentle stream of N_2 (purified by passing successively through a solution of $Ba(OH)_2$ and of water) through the cell. The pH was measured using a Radiometer TTT2

titrator and PHA 943B titration module in conjunction with a Radiometer GK2402B combined electrode, and recorded as a function of added 0.1000 M NaOH (delivered by a Radiometer ABU 12 autoburette), on a Radiometer SBR 3 titrigraph. Standard pH 4 and pH 7 buffers were used to check the electrode linearity and standardize the pH meter at the beginning of each series of experiments. The ionic strength was maintained constant by using a medium of 0.3 M in $NaNO_3$ in 50% aq. (v/v) dioxan (freshly distilled from KOH prior to use and containing no titratable acid). Data were analyzed by a computer version of the Simms method[74] and the reported pK_a's are the mean of three determinations with a precision of ± 0.01 pH unit.

ii. Metal binding constants

Stock solutions of Co^{++}, Ni^{++}, Cu^{++} and Zn^{++} were prepared from their reagent grade hydrated nitrate or chloride salts and standardized by EDTA titration. For the weakly binding ligands the pK_L[64] values were obtained by the method of Martell and Calvin,[75] using a 20-fold excess of metal ion, and analyzed by the following equation:

$$\frac{1}{K_L} = \frac{K_a^1 - K_a}{K_a\ [\text{total } M^{++}]}$$

where K_L is the dissociation constant, K_a the acid dissociation constant, $K_a{}^1$ the apparent acid dissociation constant in the presence of metal and the bracketed term the total concentration of metal ion in solution. For the stronger binding ligands a 3-4 fold excess of ligand over metal ion was titrated as before and the data analyzed by computer program,[74] the figures quoted being the average of 3 determinations.

NMR investigation of the reduction of 55

A Varian HA-100-15 spectrometer with Fourier transform modifications provided by a Digilab FTS NMR-3 system was used.

Typically 10^{-2}–10^{-1} M solutions of 55 (or other thiol) and NADH analogue were used, zinc ion was introduced by the addition of a small volume of conc. $ZnCl_2$ in D_2O.

The anaerobic experiments were carried out in the following manner. Special glass apparatus was constructed in which a thin-walled NMR tube was joined to a tube (closed at one end and with a 14/20 joint at the other) as a side arm. In one tube was 55 (or other thiol) in 0.2 mL CD_3CN and in the other the NADH analogue and Zn^{++} in 0.3 mL CD_3CN/D_2O (50/50). The two components were then subjected to 10 cycles of freeze-thawing in liquid nitrogen at $<10^{-5}$ torr. The two

solutions were then mixed in the NMR tube which was sealed under vacuum.

The isolation of products from the aerobic reduction studies on 55 were carried out as in the following example (the anaerobic cases being similar).

A reaction mixture, containing 15.6 mg (8×10^{-5} mol) 55, 17.1 mg (8×10^{-5} mol) BNAH and 8×10^{-5} mol $ZnCl_2$ in 0.5 mL 70% CD_3CN was left for 15 h and added to a solution of 5 g EDTA in 50 mL water at pH 9. The resultant mixture was continuously extracted with $CHCl_3$ for 24 h. The $CHCl_3$ extract was dried (Na_2SO_4) and solvent removed in vacuo. The NMR spectrum of the residue was similar to that in the reaction mixture (without the NAD^+ analogue). The residue was then separated by column chromatography (50 g alumina, column height 25 cm) eluting with 500 mL 4:1 CCl_4: CHCl and then $CHCl_3$. The only identifiable isolated product was the disulphide of 55 which had the following characteristics: R_f ($CHCl_3$/silca) 0.7; IR (cast) 1699, 2920 cm^{-1} (no S-H stretch at 2550 cm^{-1}); NMR (100MHz, CD_2Cl_2,FT spectrum) 1.45 (d), 2.70 (s), 2.9-3.5 (m), 7.2-7.3 (m), 7.7-7.9 (m); mass spectrum m/e (rel intensity) 194 (M^+/2,100); exact mass calcd. for $C_{10}H_{12}NOS$ 194.0640, found 194.0638.

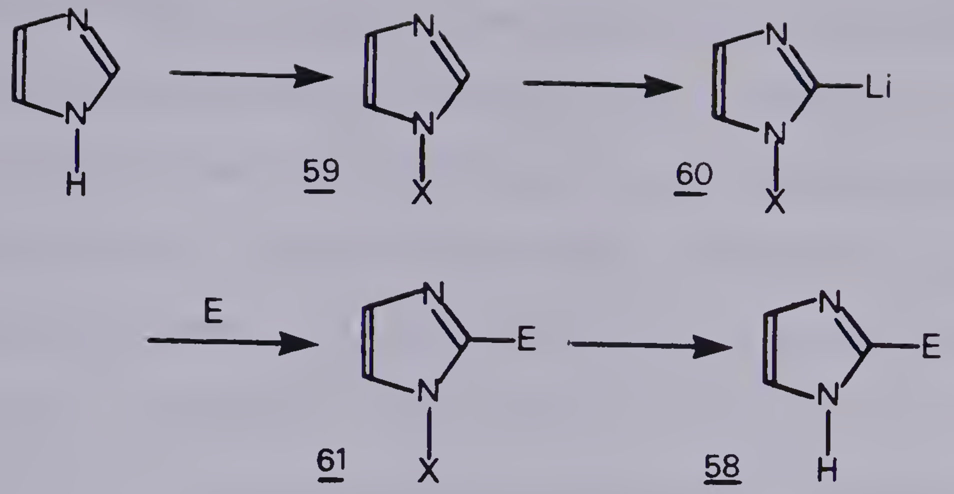
AN EASILY INTRODUCED AND REMOVED PROTECTING GROUP FOR IMIDAZOLE NITROGEN:

A CONVENIENT ROUTE TO 2-SUBSTITUTED IMIDAZOLES

INTRODUCTION, RESULTS AND DISCUSSION

The syntheses of 1-H-2-substituted imidazoles 58 can be approached from several directions. Older, classical methods involve the construction of the ring with the 2-substituent in place (e.g. Radziszewski and Weidenhagen syntheses).[92] Other routes include the dehydrogenation of the parent imidazolidine by $BaMnO_4$,[93] addition of isocyanates at high temperature,[94] modification of the 2-CF_3 derivative[95] and photochemical rearrangement of the 1-isomer[96] (this procedure also gives the 4-isomer).

More general routes, based on the use of a suitable N-protecting group are summarized in eq. 14. A protecting group is introduced onto the 1-position to give 59 which may be lithiated to give the anion 60, whose reaction[97,98] with an electrophile gives the protected product 61 which upon deblocking gives 58. For the most part however, deprotection of the imidazole nitrogen involves acidic, basic or reductive conditions and problems are often encountered in the lithiation or addition steps. For example, whilst N-benzylimidazoles can be readily prepared and the benzyl



eq. 14

group removed,[99,100] the lithiation step has been reported to give benzyl deprotonation as well as deprotonation at C-2.[101] N-Alkoxymethyl groups direct the lithiation to C-2 but their removal requires strong acid reflux[101,102] and the yields of the isolated products are modest[101,102] to nil.[103] The N-tosyl group is easily introduced[104] and removed[105] but the nucleophilicity of the corresponding C-2 anion is low.[106]

Finally, the trityl group has been shown to be a useful protecting group for imidazole itself,[107] the yields of 2-substituted products being good to excellent starting from the N-protected form. The method appears to be fairly general; however, the conditions reported for deprotection require refluxing acidic alcohol media[107] and may preclude its applicability for very acid sensitive products.

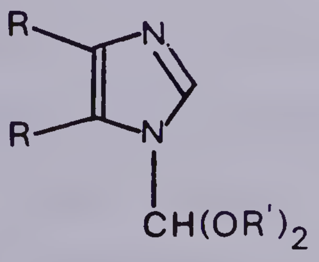
During the course of our work on imidazole containing enzyme models, we required an N-protecting group which could be easily inserted and if necessary be removed under completely neutral conditions. In particular, a synthesis of the potential carbonic anhydrase model, tris-(2-imidazoyl) phosphine was required. Although the blocked product 61 (X = ethoxymethyl, E = P/3) could be prepared,[108] deprotection was accompanied by C-P bond cleavage.

The diethoxymethyl function (59, X = $(EtO)_2CH$) meets these requirements since it is readily hydrolyzed under neutral or acid conditions in a few minutes. In addition, metallation of 62 gives only 60 (X = $(EtO)_2CH$) in less than 5 minutes, as evidenced by quenching of the reaction mixture with D_2O and examination of the NMR spectrum.

This anion is an effective nucleophile, reacting with a variety of electrophilic reagents to give good yields of the 2-substituted imidazoles after hydrolysis (see Table 4).

The protected imidazole 62 is prepared by heating a mixture of the imidazole with an excess of triethyl orthoformate in the presence of an acid catalyst with continual removal of the alcohol produced. While we have not attempted to maximize yields, a 1:4 ratio of imidazole to orthoformate routinely gives 80% yields of

the purified product. NMR analysis of the reaction mixture shows only starting material and product with no discernible disubstitution. The method is also applicable to the more sterically hindered 4,5-dimethyl and 4,5-di*iso*propyl imidazoles, protection giving 63 and 64 respectively, proceeding in good yields in both cases. 4,5-Di*iso*propylimidazole appears to be particularly hindered; N-protection with trityl fails[106b] and the orthoamide derived from triethyl orthoformate is obtained in only low yield, starting material being mostly recovered. The less bulky dimethoxymethyl group is, however, readily introduced. Using an analogous procedure, we were unable to isolate the corresponding orthoamide of benzimidazole in useful yield.



	R	R'
62	H	Et
63	CH_3	Et
64	$CH(CH_3)_2$	Me

The metallation reactions are carried out in dry ether or THF at -40°, depending upon the solubility of the anion produced. After 15 minutes the electrophilic agent is introduced and the reaction mixture allowed to come to room temperature overnight. The optimum conditions were not determined but it is apparent from

the appearance of precipitates and color changes, that the reaction is not instantaneous in some cases.

The deprotection and work-up procedures depend on the sensitivity of the product. For instance, the acid-sensitive phosphines (see table and exptl.) were isolated by stirring the crude reaction product (the tris N-protected analogues) at 25° or 56° in 5-10% aqueous acetone. Within a few minutes, crystals of the phosphines appeared, the hydrolysis being complete in a few hours.

For those products for which it is not necessary to maintain a non-acidic environment, the product imidazole is conveniently obtained by first extracting the reaction mixture with aq. HCl, neutralizing the acid extracts with solid $NaHCO_3$, and extracting the neutral aqueous mixture with $CHCl_3$. Deprotection accompanies the acid extraction.

While the isolated, overall yields were generally good, in cases where there is an acidic hydrogen present on the electrophile, the yields are lower, presumably due to proton abstraction by the 2-lithio anion.

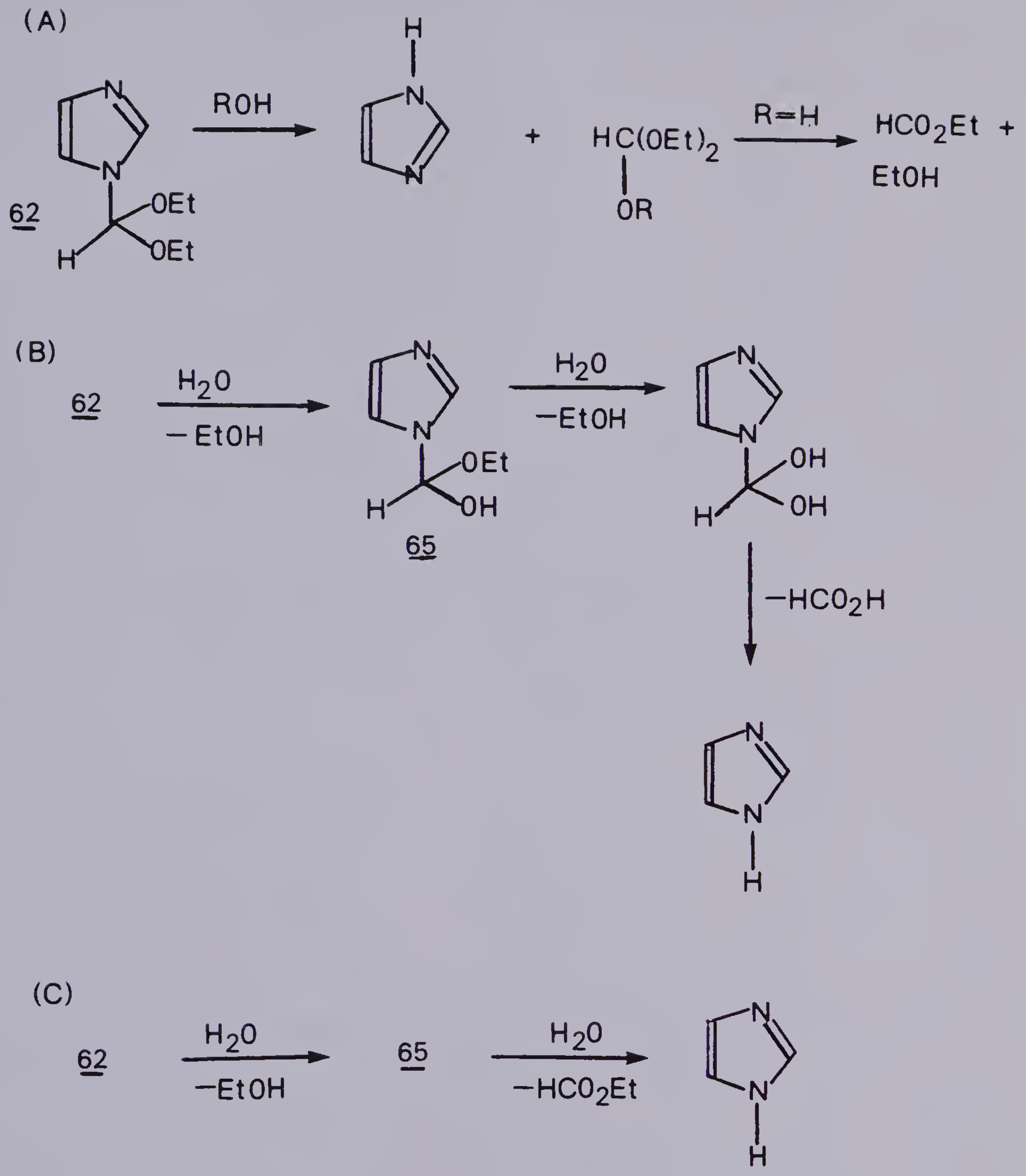
The deprotection of the protected imidazole 62 was followed by NMR in D_2O at 40°. Hydrolysis is complete in less than 30 minutes, no significant "pH" change is observed ("pH" maintained at ≈ 7) and the initial products are imidazole, ethanol and ethyl formate. The

solvolysis was also monitored in d_4-methanol and found to be complete in a few hours, the products being only orthoester and imidazole. Thus non-aqueous removal of the N-dialkoxy group is feasible.

Although it requires more kinetic analysis, the deprotection of 62 appears mechanistically interesting. Of the three possible mechanisms summarized in Scheme 7, mechanism B can be eliminated (at least in D_2O) since no formic acid is produced before free imidazole is liberated: formic acid does appear in the reaction mixture later but at the expense of ethyl formate. Inspection of the NMR aromatic region during hydrolysis of 62 in D_2O shows the presence of extra peaks indicative of some imidazole species other than 62 and imidazole itself, which may be tentatively ascribed to 65, the product of C-O rather than C-N cleavage (mech. C).

However, this assignment is based on comparison of the NMR spectra of 62 in $CDCl_3$ (see exptl.) and the reaction mixture in D_2O. In the former case the 4,5-hydrogens appear together as a multiplet at δ 7.1 and in D_2O peaks at δ 7.23, 7.11 (imidazole) and 7.08 are observed. The presence of extra peaks may be simply an effect of the different solvents used which make the 4,5-hydrogens non-equivalent

Scheme 7


(A)
62
OEt
OEt
H
ROH
H
N
N
+
HC(OEt)2
OR
R=H
HCO2Et +
EtOH
(B)
62
H2O
− EtOH
OEt
OH
H
65
H2O
−EtOH
OH
OH
H
−HCO2H
N
H
(C)
62
H2O
−EtOH
65
H2O
−HCO2Et
N
H

in aqueous solution but coincidental in $CDCl_3$. Thus, without detailed kinetic analysis we have no definite evidence either for or against mechanisms A and C.

TABLE 4. Preparation of 2-substituted imidazoles.

Reactant	Electrophile	Product		% Yield
62	CO_2	-COOH	(66)	60
62	n-C_4H_9I	-nC_4H_9	(67)	84
62	CH_3CONMe_2	-$COCH_3$	(68)	80
62	C_6H_5CHO	-$CHOHC_6H_5$	(69)	77
62	2-$C_5H_4NCOCH_3$	-$C(CH_3)OHC_5H_4$	(70)	64
62	Fluorenone	-9-hydroxy fluorenyl	(71)	84
62	Cyclohex-2-enone	1-hydroxy cyclohex-2-enyl	(72)	49
62	$(C_6H_5)_2CO$	-$C(OH)(C_6H_5)_2$	(73)	72
62	PCl_3	-P/3	(74)	36
63	$HCONMe_2$	-CHO	(75)	82
63	PCl_3	-P/3	(76)	46
64	PCl_3	-P/3	(77)	55

EXPERIMENTAL

Routine spectral measurements were performed as described before.

N-(1,1-diethoxymethyl)imidazole[109] (62)

Imidazole (12.8 g, 0.2 mol), 118.4 g (0.8 mol) of triethylorthoformate and 1 g of p-toluenesulphonic acid were heated at 130° until no more ethanol was distillable from the reaction mixture. The excess orthoformate was removed in vacuo, 1 g of solid Na_2CO_3 was added and the residue fractionally distilled to give 28.2 g (82%) of the product as a colorless oil: bp 52° (0.02 torr); IR (film) 1060, 2960 cm^{-1}; NMR (60 MHz, $CDCl_3$) δ1.22 (t,6H), 3.59 (q,4H), 6.06 (s,1H), 7.1 (m,2H), 7.7 (m,1H); mass spectrum m/e (rel intensity) 170 (M^+,6), 103 (100); exact mass calcd. for $C_8H_{14}N_2O_2$ 170.1055, found 170.1052.

N-(1,1-diethoxymethyl)-4,5-dimethylimidazole[109] (63)

This was prepared in 77% yield from 4,5-dimethylimidazole[110] in an analogous fashion to 63 and isolated as a colorless oil: bp 82-7° (0.5 torr); IR (film) 1070, 2980 cm^{-1}; NMR (60MHz,$CDCl_3$) δ1.22 (t,6H), 2.13 (s,3H), 2.17 (s,3H), 3.57 (q,4H), 5.90 (s,1H), 7.57 (s,1H), mass spectrum m/e (rel intensity) 198 (M^+,25),

156 (26), 103 (100); exact mass calcd. for $C_{10}H_{18}N_2O_2$ 198.1368, found 198.1364.

N-(1,1-dimethoxymethyl)-4,5-di*iso*propylimidazole[109] (*64*)

4,5-Di*iso*propylimidazole[110] (10 g, 0.067 mol), 27 g (0.25 mol) of trimethyl orthoformate and 0.2 g of p-toluene sulphonic acid were refluxed in 100 mL of toluene until 100 mL of distillate had collected over 6 H. The mixture was cooled and 2.7 g of starting imidazole was recovered by filtration. The filtrate was fractionally distilled to give 8.8 g (84% based on recovered starting material) of the product as a colorless oil: bp 96-100° (0.5 torr); IR (film) 1060, 2970 cm^{-1}; NMR (60 MHz,$CDCl_3$) δ1.26 (d,6H), 1.30 (d,6H), 3.33 (s,6H), 2.80-3.43 (m,2H), 5.86 (s,1H), 7.61 (s,1H); exact mass calcd. for $C_{12}H_{22}N_2O_2$ 226.1681, found 226.1681.

Preparation of the lithium anions of the N-protected imidazoles

To 50 mL of dry THF or ether kept under N_2 at -40° and containing 0.02 mol of the appropriate N-protected imidazole was added via syringe 0.02 mol of n-BuLi in hexane such that the temperature did not rise above -35°.

After the addition, the pale yellow solution was left for 15 min. at -40° before use.

Imidazole-2-carboxylic acid (66)

To 0.02 mol of the lithium anion of 62, in 50 mL THF, was added 5 g (0.11 mol) of crushed CO_2, via a solid addition tube, over a period of 10 min. After stirring for 15 min., 1 mL of water was added and the mixture left to stir and warm to room temperature for 16 h. The resultant precipitate was isolated by decanting the supernatant. The white solid was dissolved in 5 mL water and the solution cooled in ice. Acidification of this solution to pH 3 by 3 M HCl gave 1.34 g (60%) of the product as white flakes after filtration: mp 172-4°, lit.[111] 163-4°; IR (KBr disc) 1400, 1640 cm^{-1}; NMR (60MHz,D_2O) δ7.57 (s); mass spectrum m/e (rel intensity) 112 (M^+,100), 68 (94); exact mass calcd. for $C_4H_4N_2O_2$ 112.0273, found 112.0269.

2-n-Butylimidazole (67)

To 0.02 mol of the lithium anion of 62, in 50 mL THF, was added 2.73 mL (0.024 mol) of n-BuI over 5 min. and the mixture left to warm to room temperature with stirring. After 20 h, 50 mL of ether was added and the reaction mixture extracted with 0.1 M HCl (4 x 50 mL). The combined acid extracts were neutralized by addition of solid $NaHCO_3$ and this solution extracted with $CHCl_3$ (6 x 100 mL). The combined $CHCl_3$ extracts were dried

(Na_2SO_4) and the solvent removed in vacuo. Kügelrohr distillation of the resultant oil (oven temp. 93°, 0.02 torr) gave 2.08 g (84%) of 67 as a colorless oil which gelled on cooling: IR (cast) 1400, 2900 (br) cm^{-1}; NMR (60MHz,$CDCl_3$) δ0.8-2.0 (m,7H), 2.78 (t,2H), 6.98 (s,2H), NH not apparent; mass spectrum m/e (rel intensity) 124 (M^+,16), 82 (100); exact mass calcd. for $C_7H_{12}N_2$ 124.1000, found 124.0998.

Anal. calcd. for $C_7H_{12}N_2$: C,67.70; H,9.74; N, 22.56. Found: C,67.75; H,9.76; N,22.38.

2-Acetylimidazole (68)

To 0.02 mol of the lithium anion of 62, in 50 mL THF, was added 2.79 mL (0.03 mol) of CH_3CONMe_2 over 5 min. and the mixture left to warm up and stir for 12 h. Ether (50 mL) was then added and the mixture extracted with 0.1 M HCl (4 x 50 mL). The combined acid extracts were neutralized with solid $NaHCO_3$ and this solution extracted with $CHCl_3$ (8 x 100 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and solvent removed in vacuo to give a solid which was recrystallized from benzene/CH_3OH yielding 1.77 g (80%) of 68 as white flakes: mp 136-7°, lit.[96] 137-7.5°; IR (KBr disc) 1410, 1680 cm^{-1}; NMR (60MHz,CD_3OD) δ2.58 (s,3H), 7.30 (m,2H); mass spectrum m/e (rel intensity) 110 (M^+,100), 95 (58); exact mass calcd. for $C_5H_6N_2O$ 110,0480, found 110.0479.

1-(2-imidazolyl)-1-phenylmethanol (69)

To 0.02 mol of the lithium anion of 62, in 50 mL THF, was added 2.44 mL (0.024 mol) of benzaldehyde over 5 min. and the solution left to warm to room temperature with stirring. After 12 h, 50 mL of ether was added and the mixture extracted with 0.1 M HCl (4 x 50 mL). The combined acid extracts were neutralized with solid $NaHCO_3$ and this solution extracted with $CHCl_3$ (6 x 100 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and the mixture evaporated to a small volume from which 2.68 g (77%) of the product was collected, by filtration, as white flakes mp 205-6°, lit.[113] 199-201°; IR (cast) 530, 760, 1060, 3200 (br) cm^{-1}; NMR (60MHz,CD_3OD) δ5.86 (s,1H), 6.95 (s,2H), 7.2-7.6 (m,5H); mass spectrum m/e (rel intensity) 174 (M^+,100), 156 (66); exact mass calcd. for $C_{10}H_{10}N_2O$ 174.0792, found 174.0792.

1-(2-imidazolyl)-1'-(2-pyridyl)ethanol (70)

To 0.02 mol of the lithium anion of 62, in 50 mL THF, was added 2.69 mL (0.024 mol) of 2-acetylpyridine over 5 min and the solution left to warm up with stirring. After 12 h, 50 mL of ether was added and the mixture extracted with 0.1 M HCl (4 x 50 mL). The combined acid extracts were dried (Na_2SO_4) and the

solvent and excess 2-acetylpyridine removed in vacuo. The resultant solid was recrystallized from ether/ $CHCl_3$ to give 2.43 g (64%) of 70 as white crystals: mp 136-7°; IR (cast) 1090, 3260 (br) cm^{-1}; NMR (60MHz, CD_3OD) δ1.92 (s,3H), 6.92 (s,2H), 7.1-7.9 (m,3H), 8.5 (m,1H); mass spectrum m/e (rel intensity) 189 (M^+,31), 174 (100), 111 (54), 78 (50); exact mass calcd. for $C_{10}H_{11}N_3O$ 189.0902, found 189.0900.

Anal. calcd. for $C_{10}H_{11}N_3O$: C,63.48; H,5.86; N, 22.21. Found: C,63.28; H,5.81; N,22.39.

9-hydroxy-9'-(2- imidazolyl)-fluorene (71)

To 0.02 mol of the lithium anion of 62, in 50 mL THF, was added a solution of 4.32 g (0.024 mol) of fluorenone dissolved in 20 mL THF, over 10 min. and the solution left to stir and warm up. After 16 h, ether (50 mL) was added and the mixture extracted with 0.1 M HCl (4 x 50 mL). The combined acid extracts were neutralized with solid $NaHCO_3$ and this solution extracted wtih $CHCl_3$ (6 x 100 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and solvent removed in vacuo. The resultant solid was recrystallized from $CHCl_3$/methanol yielding 4.18 g (84%) of the product as white crystals: mp 203-4° d; IR (KBr disc) 730, 750 cm^{-1}; NMR (60MHz,DMSO) 6.83 (s,2H), 7.1-8.1 (m,9H), NH not apparent; mass spectrum m/e (rel intensity) 248

(M^+,58), 230 (100); exact mass calcd. for $C_{16}H_{12}NO$ 248.0949, found 248.0941.

Anal. calcd. for $C_{16}H_{12}NO$: C,77.40; H,4.87; N, 11.28. Found: C,77.25; H,4.76; N,11.52.

1-(2-imidazolyl)cyclohex-2-enol (72)

To 0.02 mol of the lithium anion of 62, in 50 mL THF, was added 2.32 mL (0.024 mol) of cyclohex-2-enone over 5 min. and the mixture left to warm up with stirring. After 12 h, 50 mL of ether was added and the mixture extracted with 0.1 M HCl (4 x 50 mL). The combined acid extracts were neutralized with solid $NaHCO_3$ and then extracted with $CHCl_3$ (12 x 100 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and the solvent removed in vacuo. Recrystallization of the resultant solid from ether/methanol gave 1.61 g (49%) of the product as white crystals: mp 177-8; IR (cast) 3200 (br) cm^{-1}; NMR (60MHz,CD_3OD) δ1.6-2.3 (m,6H), 5.7-6.1 (m,2H), 6.93 (s,2H); mass spectrum m/e (rel intensity) 164 (M^+, 22), 145 (100); exact mass calcd. for $C_9H_{12}N_2O$ 164.0949, found 164.0949.

Anal. calcd. for $C_9H_{12}N_2O$: C,65.83; H,7.37; N, 17.06. Found C,65.72; H,7.31; N,17.39.

Imidazol-2-yl diphenylmethanol (73)

To 0.02 mol of the lithium anion of 62, in 50 mL

THF, was added a solution of 4.40 g (0.024 mol) benzophenone in 20 mL THF, over 5 min. and the mixture left to warm up with stirring. After 16 h, 50 mL ether was added and the mixture extracted with 0.1M HCl (4 x 50 mL). The combined acid extracts were neutralized with solid $NaHCO_3$ and then extracted with $CHCl_3$ (4 x 100 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and solvent removed in vacuo. Recrystallization of the resultant solid gave 3.61 g (72%) of 73 as white crystals: mp 195-7°, lit.[114] 189-90°; IR (cast) 700, 760, 3250 (br) cm^{-1}; NMR (60MHz,d_6DMSO) δ6.5 (s,1H), 6.94 (s,2H), 7.1-7.6 (m,10H), NH not apparent; mass spectrum m/e (rel intensity) 250 (M^+,100), 173 (62); exact mass calcd. for $C_{16}H_{14}N_2O$ 250.1106, found 250.1106.

Tris-(2-imidazolyl)phosphine (74)

To 0.0966 mol of the lithium anion of 62, in 300 mL ether, was added 4.42 g (0.0322 mol) of freshly distilled PCl_3 and the mixture left to warm up with stirring. After 24 h, 60 mL conc. ammonia was added and the mixture stirred for 30 min. The ether layer was then separated, dried (Na_2SO_4) and solvent removed in vacuo. The resultant residue was then dissolved in 100 mL of 5% aq. acetone and stirred overnight. The resultant precipitate was collected by filtration and recrystallized from methanol/ether to give 2.4 g (36%)

of 74 as white crystals: mp 229-31°; IR (KBr disc) 760, 1100, 3000 (br) cm^{-1}; NMR (60MHz,DMSO) δ7.24 (s); mass spectrum m/e (rel intensity) 232 (M^+,7), 68 (100); exact mass calcd. for $C_9H_9N_6P$ 232.0626, found 232.0625.

Anal. calcd. for $C_9H_9N_6P$: C,46.55; H,3.88; N,36.21. Found: C,46.45; H,3.98; N,35.99.

4,5-Dimethylimidazol-2-ylcarbaldehyde (75)

To 0.01 mol of the lithium anion of 63, in 25 mL THF, was added 0.99 mL (0.015 mol) of dry DMF over 5 min and the mixture left to warm to room temperature with stirring. After 12 h, 25 mL of ether was added and the mixture extracted with 0.1 M HCl (4 x 25 mL). The combined acid extracts were neutralized with solid $NaHCO_3$ and extracted with $CHCl_3$ (4 x 50 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and solvent removed in vacuo. The resultant solid was recrystallized from ether/methanol to give 0.88 g (82%) of the product as white crystals: mp 164-5°; IR (cast) 805, 1675 cm^{-1}; NMR (60MHz,$CDCl_3$) δ2.30 (s,6H), 9.63 (s,1H); mass spectrum m/e (rel intensity) 124 (M^+,100), 95 (16); exact mass calcd. for $C_6H_8N_2O$ 124.0637, found 124.0637.

Anal. calcd. for $C_6H_8N_2O$: C,58.05; H,6.50; N,22.56. Found: C,57.77; H,6.42; N,22.32.

Tris-(4,5-dimethylimidazol-2-yl)phosphine 76

This was prepared analogously to 74 except that the deprotection was carried out by refluxing in 10% aq. acetone for 1 h. After cooling at -20°, a 46% yield of the product (as a monohydrate) was obtained as white crystals: mp 255-7; IR (KBr disc) 1600, 3400 (br); NMR (60MHz,d_6DMSO) δ1.93 (s); mass spectrum m/e (rel intensity) 316 (M^+,77), 221 (100); exact mass calcd. for $C_{15}H_{21}N_6P$ 316.1565, found 316.1566.

Anal. calcd. for $C_{15}H_{21}N_6P \cdot H_2O$: C,53.89; H,6.88; N,25.15. Found: C,53.61; H,6.76; N,25.45.

Tris-(4,5-diisopropylimidazol-2-yl)phosphine 77

This was prepared analogously to 76 in 55% yield as white crystals after recrystallization from ethanol/water: mp 185-7.5°; IR (cast) 2960 cm^{-1}; NMR (60MHz, CD_3OD) δ1.22 (d,36H), 3.00 (m,6H); mass spectrum m/e (rel intensity) 484 (M^+,58), 301 (74), 137 (100); exact mass calcd. for $C_{27}H_{45}N_6P$ 484.3438, found 484.3438.

Anal. calcd. for $C_{27}H_{45}N_6P$: C,66.90; H,9.36; N, 17.30. Found: C,66.90; H,9.20; N,17.11.

TRIS-(4,5-DIISOPROPYLIMIDAZOL-2-YL)PHOSPHINE: A MODEL FOR THE ACTIVE SITE OF CARBONIC ANHYDRASE

INTRODUCTION

Carbonic anhydrase (CA) is a widely distributed zinc-containing metallo-enzyme[18a,c,d,114] whose only known physiological purpose is to catalyze the interconversion of carbon dioxide and bicarbonate[114d] (eq. 14).

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \qquad \text{eq. 14}$$

The enzyme, has however, been shown to catalyze other processes involving nucleophilic attack of oxygen at an electrophilic centre.[18c] These include hydration of aldehydes,[115a] pyruvic acid[115b] and alkyl pyruvates,[115c] and hydrolysis of some carboxylic, carbonic, sulphonic and phosphonic esters.[116] The fundamental importance to both plant and animal processes such as photosynthesis, calcification, pH maintenance, ion transport and CO_2 exchange[117] has led to various forms of CA being widely studied. Based on these studies a number of mechanisms for enzyme action has been proposed[29] that are compatible with most of the available physicochemical information.

The structures of human CA isozymes B and C have been determined by X-ray crystallography[118] and both show the zinc ion to be coordinated between three

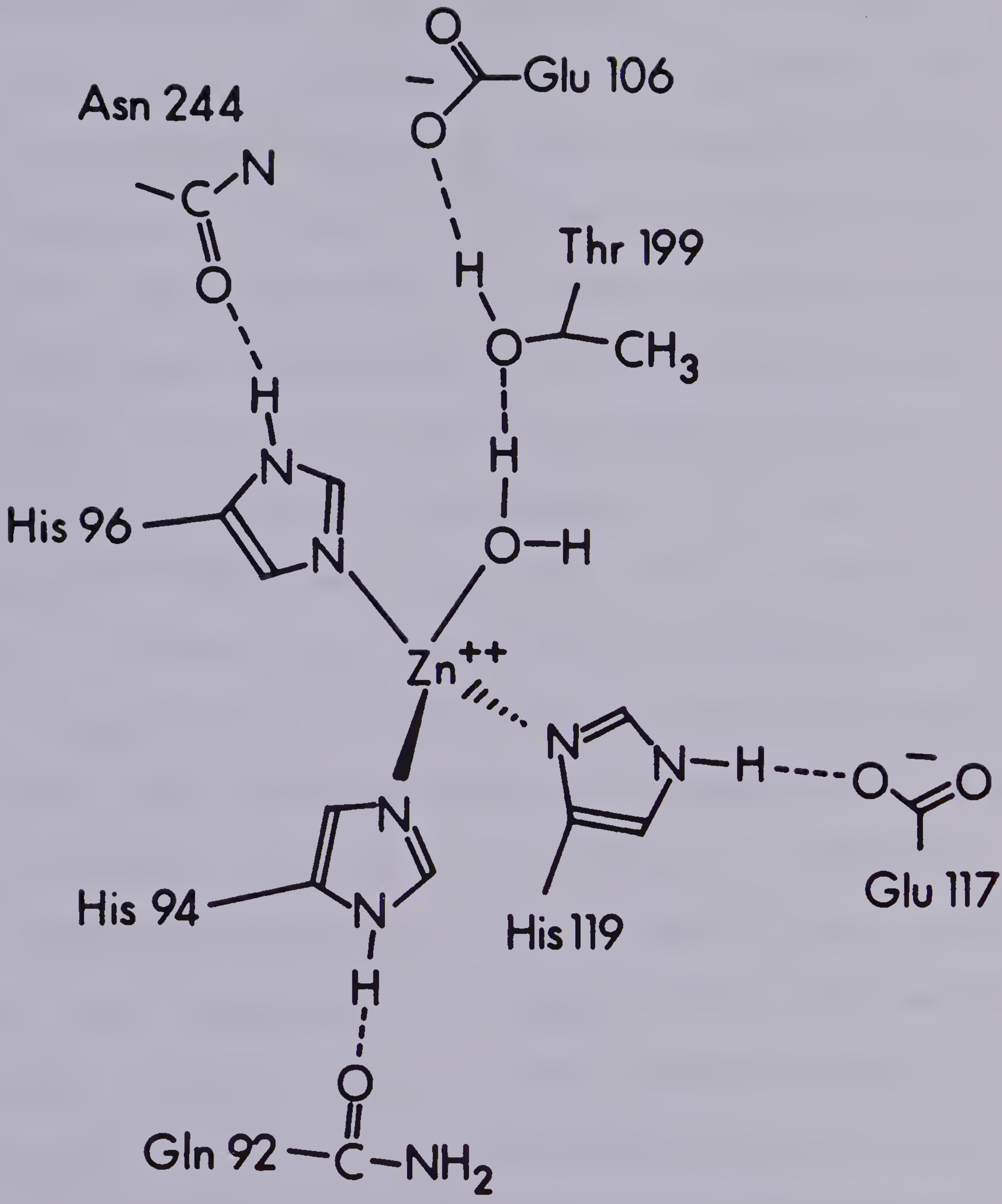

Asn 244
Glu 106
Thr 199
His 96
Zn++
His 94
His 119
Glu 117
Gln 92

Fig. 6. Schematic representation of the active site of carbonic anhydrase showing the Zn^{++} binding site and H-bonding network (ref.114e).

histidine imidazoles, the fourth ligand site is said to be occupied by a water molecule, completing a distorted tetrahedral environment (Fig. 6).

The activity of CA is influenced by one or more ionizing groups with a pK_a of around 7,[18,114] the higher pH form is more active for the hydration of CO_2 and the lower pH form in the dehydration of HCO_3^-. Four alternatives have been proposed for this ionizing group.[29] These comprise: 1. a zinc-bound water molecule. 2. a zinc-bound imidazole. 3. an imidazolium group in the active site cavity. 4. the carboxyl group in Glu-106, also in the active site pocket. At least nine mechanisms[29] have been suggested, based on these proposals. Although these mechanisms vary greatly,[29] they can be divided into two classes. In one class are mechanisms which include the ionization of a group bound directly to Zn^{++}, i.e. a water or imidazole. This ionized group gives rise to attack of CO_2 by hydroxyl via a nucleophilic or general base mechanism to give HCO_3^- (vide infra). In the second class of mechanisms, the zinc ion only plays a passive role and serves as a template on which the reaction occurs. Thus in these cases, the activity ofCA requires the correct protein structure in the active site cavity in addition to tetrahedrally bound zinc ion as in the former class.

These mechanisms have been reviewed[29] and apart

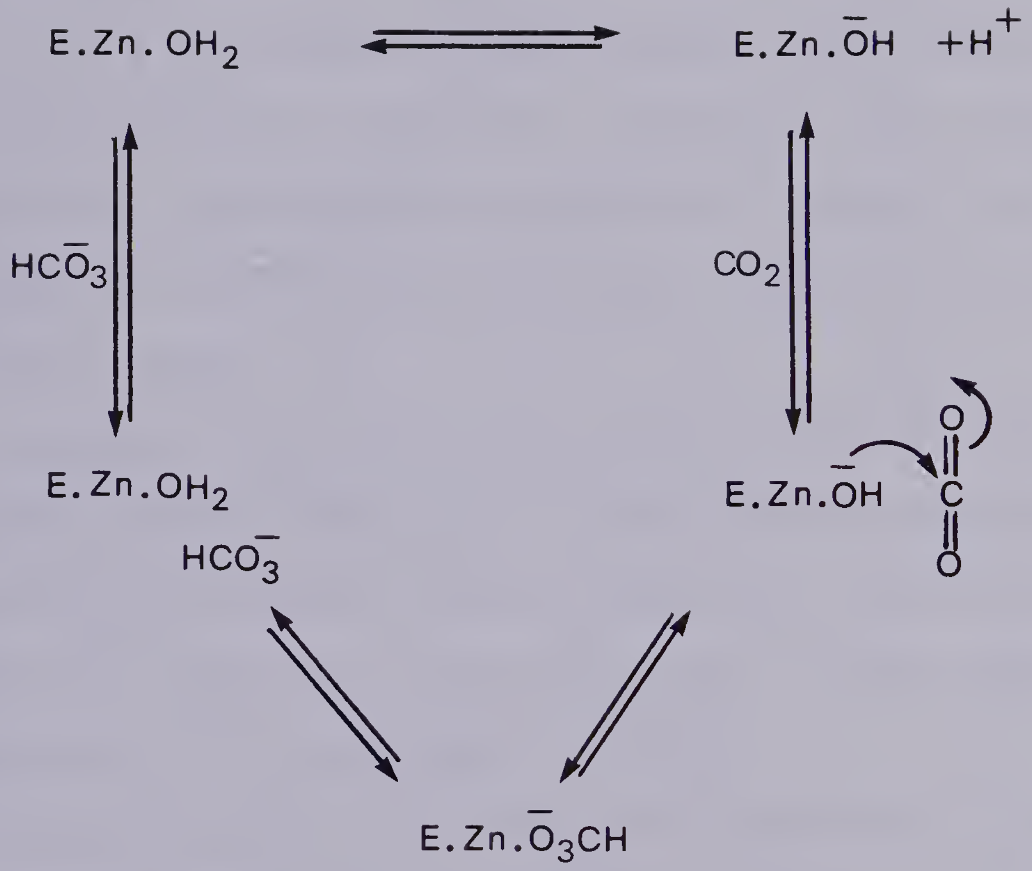
from the following example will not be discussed further.

One proposal, from the former class of mechanisms, the "bound hydroxyl mechanism",[29] is shown in Scheme 8. In this mechanism, the ionizing group is the coordinated water molecule (vide supra), which on titration gives rise to a bound hydroxyl which attacks CO_2 as shown. The resultant bound bicarbonate complex is then hydrolyzed and HCO_3^- released.

This is one of the simpler mechanisms proposed, others are more exotic and include complex hydrogen-bonded systems involving water molecules and some of the amino acid residues in the cavity.[29]

The zinc ion in CA can be removed and replaced by cobalt (II) to give a catalytically viable species and the visible spectrum of cobalt CA has been widely studied. The observed spectrum at high pH (9) shows several fairly intense (maximal ε = 300-400 M^{-1} cm^{-1}) maxima in the range 500-650 nm.[119] This has been interpreted in terms of a distorted geometry around Co^{++} corresponding to 4 and/or 5-fold coordination. The magnitude of these peaks has been tied, as has the catalytic activity toward CO_2 hydration, to the ionization of one or more functional groups with pK_a around 7, such that the intensity of color (cobalt CA is a reddish-blue) increases with pH.[120]

Scheme 8


E.Zn.OH$_2$
E.Zn.$\bar{O}$H
+H$^+$
HC$\bar{O}_3$
CO$_2$
E.Zn.OH$_2$
HC$\bar{O}_3$
E.Zn.$\bar{O}$H
O
C
O
E.Zn.$\bar{O}_3$CH

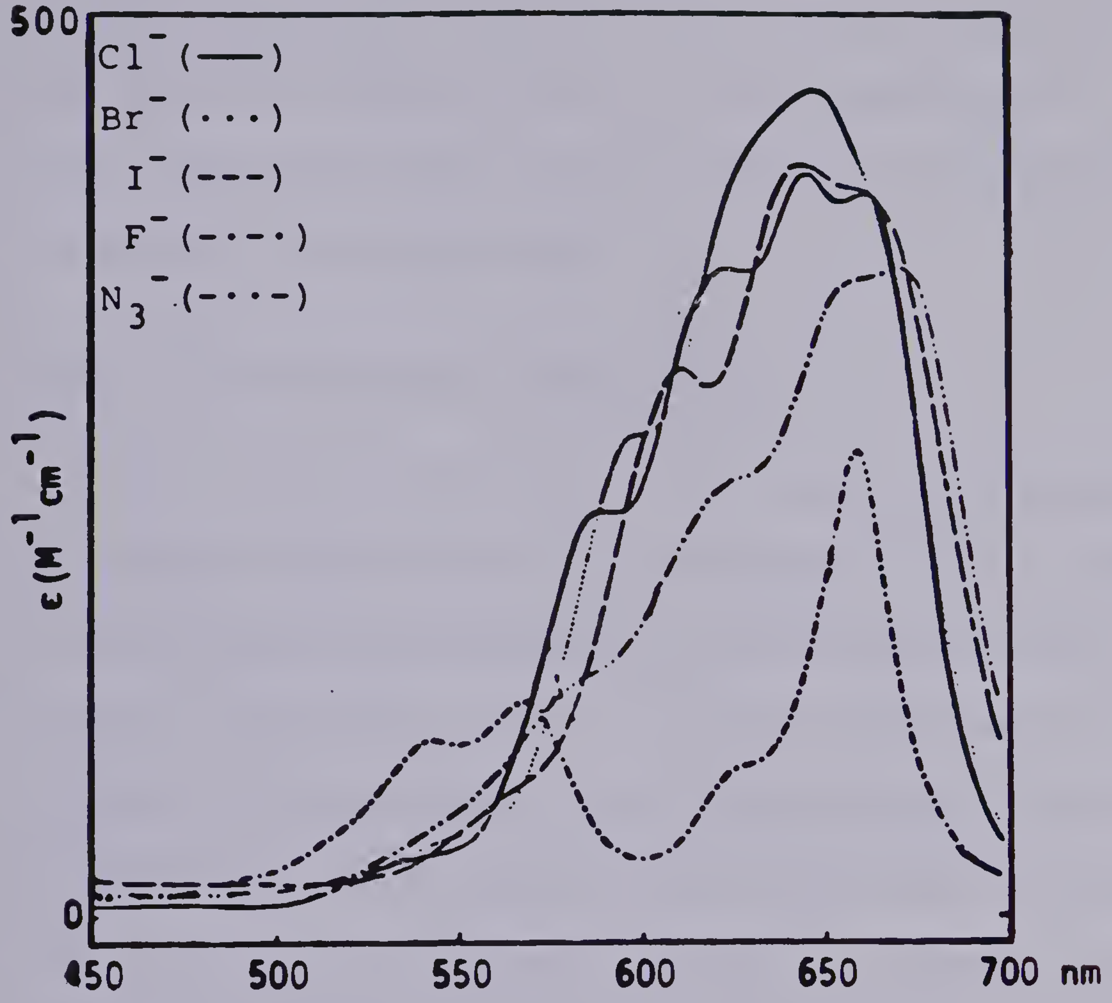
A number of small molecules has been studied as M^{++}/M^{+++} coordinated systems in order to investigate enzyme functions such as catalysis,[121] Co^{++} spectral and assorted physico-chemical[101,123] properties. However, of these, only a few[101,122a,123a,b] of the models contain approximations for the known binding site in CA.

In preliminary work[123a] it was shown that 77, tris-(4,5-diisopropylimidazol-2-yl)phosphine formed a 1:1 complex with Zn^{++} which catalyzed the process of eq. 14 in a 80% ethanol water medium (this solvent was used for solubility reasons). However, under similar conditions, no evidence was seen for catalysis of hydrolysis of p-nitrophenyl acetate, process which CA can also effect.[116]

Spectral analysis of the cobalt (II) 77 complexes revealed that in some cases, depending on counterion,[123a] species were formed in which the cobalt had coordination numbers of less than 6 as is the case in the cobalt form of CA. This is shown in Fig. 7.

The analogous less hindered phosphines 74 and 76 did not catalyze CO_2 equilibration in water nor did the visible spectra of their cobalt (II) complexes show any evidence[123a] for 4 and/or 5 coordinate Co^{++}. It was thus concluded that in order to be a model for CA a bulky ligand system should be used which ensures coordination numbers of less than 6 for the metal ion.

Co^{II}: Phosphine Absorption Spectra



Co^{II}: Enzyme Visible Absorption Spectra

S. Lindskog, Struct. Bonding (Berlin), 8, 153 (1970).

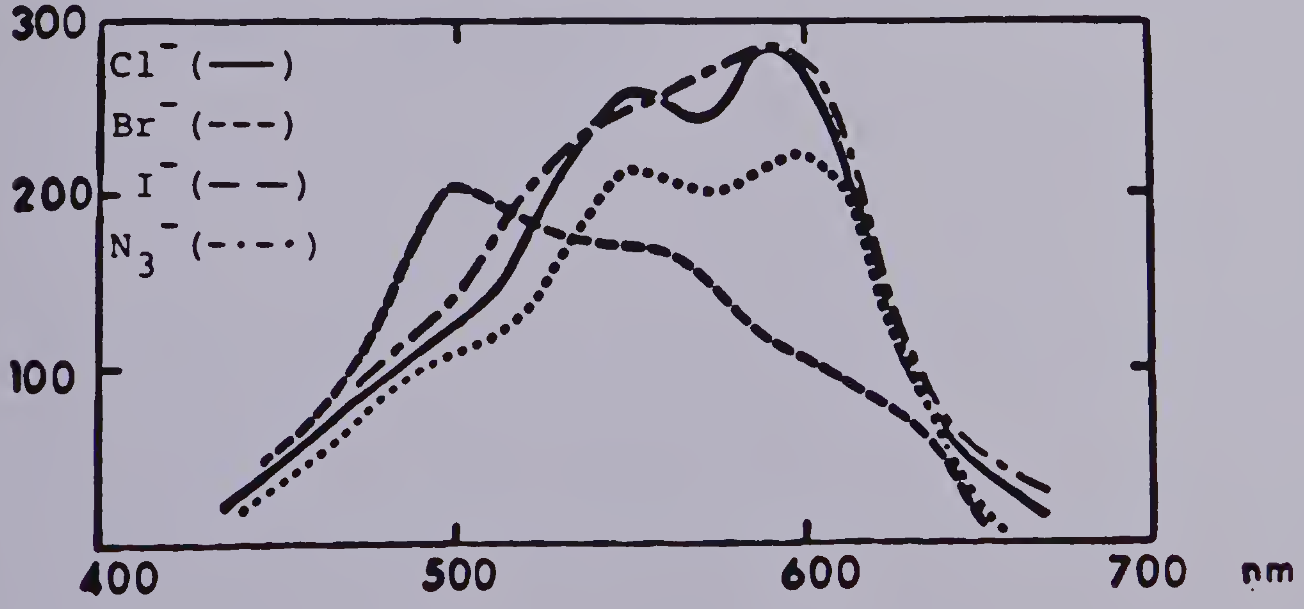


Fig. 7. Anion dependent visible spectra of 77:Co^{II} determined in 80% ethanol-H$_2$O solution saturated with Na X (X = I,Br,Cl,F), compared with that of Co^{II}:CA.

In addition, the structure of the Zn^{++}. 77. $2Cl^-$ complex was determined by X-ray crystallography[124] and was found to contain the zinc ion coordinated by the three imidazoles and one chloride ion in a pseudo-tetrahedral configuration.

Scope and purpose of this research

The purpose of the present work is to extend the preliminary work of Brown and Huguet[123a] and investigate in more detail the catalytic properties of the Zn^{++} and Co^{++} complexes of 77. In particular we were interested in determining the binding constants of Zn^{++} and Co^{++} with 77, studying the effect of pH on the catalysis of CO_2 hydration and HCO_3^- dehydration, catalytic inhibition by monovalent anions and the effect of substituting Co^{++} for Zn^{++}, both on the spectral and catalytic properties.

RESULTS AND DISCUSSION

Binding constants

The pK_a values[74] for 77 and the binding constants[74] of 77 with Zn^{++} and Co^{++} were determined using 5×10^{-3} M solutions of ligand in 80% ethanol-water with an ionic strength of 0.2 M, adjusted with $NaClO_4$ or NaCl. The data were analyzed as described before.[74] For the binding constants, the method for strong binders was used. The results are shown in Table 5.

TABLE 5. pK_a and binding constant data for 77.

	$NaClO_4$[a]	NaCl[b]
pK_{a_3}	2.55 ± 0.03	3.12 ± 0.02
pK_{a_2}	4.36 ± 0.01	4.43 ± 0.04
pK_{a_1}	6.67 ± 0.07	6.45 ± 0.05
$pK_L(Co^{++})$	3.48 ± 0.08	4.30 ± 0.15
$pK_L(Zn^{++})$	6.00 ± 0.04	7.71 ± 0.06

a. 0.2 M $NaClO_4$, 80% ethanol, 25°C.

b. 0.2 M NaCl, 80% ethanol, 25°C.

It can be seen that both the pK_a and pK_L values depend on the counterion used. For the pK_a data, the

variations may be due to changes in the structure of the solvent, caused by the affect of the different anions (ClO_4^- and Cl^-). For the pK_L data, the differences are possibly caused by the formation of stable ternary complexes of the form M^{++}. 77. Cl^- in the latter case, since pK_L values are invariably larger when Cl^- is a ligand (c.f. the crystal structure of Zn^{++}. 77. $2Cl^-$, vide supra). Whereas a computer method has shown it is possible to replace coordinated Cl^- in this case by ClO_4^- some molecular reorganization is necessary to effect this which may cause weaker binding (or no binding at all) between the binary complex Zn^{++}. 77. and ClO_4^-. In the cobalt case, using Cl^- as counterion, blue solutions are formed whose visible spectra correspond to four coordinate Co^{++}. However, with ClO_4^- as counterion no indication of four or five coordinate Co^{++} is seen from the spectrum, only weak absorption due to octahedrally coordinated Co^{++} being observed, suggesting that Cl^- is an integral component of the tetrahedral complex.

It should be noted that in both cases, the Co^{++} binding constant is significantly smaller than the Zn^{++} case, which parallels the behavior in the enzyme[125] ($pK_L(Zn^{++})$ 10.5, $pK_L(Co^{++})$ 7.2). In this case the difference in size of the metal ions and the propensity for four (or five) coordination may be important.

Kinetic studies

i. Method

The following procedure was used to assess the catalysis of the compounds under investigation.

The kinetics were monitored under pseudo-first order conditions with $[H^+]$ being held constant by HEPES or MES buffers. Of course, $[H^+]$ must vary to some extent since this provides the method for observing the net reaction (vide infra), however under appropriate buffering conditions, $\Delta[H^+]$ can be held to <8%, corresponding to a measured pH change of <0.04 pH units. Under these conditions eq. 14 reduces to a typical first order equilibrium reaction eq. 15:

$$CO_2 \underset{k_{dehyd}}{\overset{k_{hyd}}{\rightleftharpoons}} HCO_3^- \qquad \text{eq. 15}$$

in which $dx/dt = k_{obs}\,(Xe-X)$ where X and Xe denote the concentration of the product of the reaction (HCO_3^- for the hydration, CO_2 for the dehydration) at time t and at equilibrium respectively. Thus, $k_{obs} = (k_{hyd} + k_{dehyd})$, the sum of all the forward and reverse rate constants which includes the various forms of the buffer and other species present.

Since neither CO_2 nor HCO_3^- have easily monitored

spectral properties, a well established indicator technique[126] was used in which the small change in $[H^+]$ is monitored by observing a parallel rapid acid-base reaction in which the concentration of an intensely colored indicator anion changes as $[H^+]$ varies (eq. 16).

$$A^- + CO_2 + H_2O \underset{}{\overset{\text{slow}}{\rightleftharpoons}} HCO_3^- + H^+ + A^- \underset{}{\overset{\text{fast}}{\rightleftharpoons}} HCO_3^- + HA \quad \text{eq. 16}$$

The time scale of the reaction was found to be suitable for stopped flow kinetics and a typical experiment was performed as follows. In one syringe was placed a solution of 10^{-4} M indicator (BCP, bromocresol purple), 5 x 10^{-2} M buffer, adjusted to the appropriate "pH reading" by addition of M NaOH and the calculated amount of solid $NaClO_4$ to bring the ionic strength to 0.2 M. The second syringe contained a solution of 10^{-3} M $NaHCO_3$ for the dehydration reaction, (for the hydration reaction a saturated 80% ethanol-water solution was prepared and diluted 5 fold), and the appropriate amount of solid $NaClO_4$ for an ionic strength of 0.2 M. When used, the catalyst and inhibitors were added to the first syringe.

ii. Control experiments

The following control experiments were performed to ensure the catalysis observed was due to complex

TABLE 6. Control Experiments for $HCO_3 + H^+ \rightarrow$ Dehydration Reaction. pH 6.40 (HEPES)[a]

Run #	[Indicator][b]	[phosphine][c]	$[Zn^{++}]$[d]	k_{obs} (sec^{-1})
1	5×10^{-5} M.	0	0	0.822±0.007
2	5×10^{-5} M.	5×10^{-4} M.	0	0.833±0.006
3	5×10^{-5} M.	0	5×10^{-4} M.	0.831±0.004
4	5×10^{-5} M.	5×10^{-4} M.	5×10^{-4} M.	1.272±0.025
5	2×10^{-4} M.	5×10^{-4} M.	5×10^{-4} M.	1.277±0.008

a. T = 25±0.2°C; pH change < 0.04 units, monitored at 600 nm. Ionic strength = 0.2 $NaClO_4$.

b. Bromocresol purple.

c. tris-2[4,5-diisopropylimidazolyl]phosphine.

d. $Zn(ClO_4)_2$

and not metal or phosphine alone (see Table 6).

In the presence of added Zn^{++} but no 77, and vice versa, no catalysis was observed for HCO_3^- dehydration. When the kinetics were run in the absence of substrate (CO_2 or HCO_3^-) only a rapid dilution effect was seen, verifying the assumption that the indicator acid-base reaction is rapid relative to CO_2/HCO_3^- interconversion. Increasing the concentration of BCP caused no change in the observed rate constant.

iii. Catalysis of HCO_3^-/CO_2 equilibrium with Zn^{++}. 77

The rate constants determined from these experiments are shown in Tables 7 (for approaching equilibrium from excess CO_2) and 8 (from excess HCO_3^-). The kinetics were performed over a range of pH 6.1 to 7.3 and representative examples of the data are plotted in Fig. 8.

The data show that for both processes, the observed rate constants in the presence of the Zn^{++}-catalyst were significantly larger than in its absence. A linear relationship is seen for the increase of observed rate constant with catalyst concentration, indicating that this increase is due to a bimolecular interaction between catalyst and HCO_3^-/CO_2, the rate enhancement being first order in catalyst.

The relative magnitude of the catalytic effects

TABLE 7 . Pseudo First Order Rate Constants for $CO_2 + H_2O \rightarrow$ Hydration[a,b]

	k_{obs} (sec^{-1})			k_{cat}
	[Catalyst] x 10^4 M.			(M^{-1} sec^{-1})[d]
pH	[0]	[2.5]	[5.0]	
7.30 (HEPES)	0.235±0.002	0.380±0.002	0.548±0.003	626±10
7.00 (HEPES)[c]	0.446±0.003	0.635±0.006	0.818±0.014	744±34
6.70 (HEPES)	0.733±0.008	0.917±0.004	1.118±0.012	770±40
6.40 (HEPES)	1.263±0.028	1.539±0.026	1.712±0.031	898±118

a. Followed by observing change in absorbance of bromocresol purple at 600 nm; T = 25±0.2°; Ionic strength = 0.2 $NaClO_4$, 80% $EtOH/H_2O$ (v/v).

b. pH changes < 0.04 units; 2.5 x 10^{-2} M. buffer.

c. CO_2 solutions made by dilution of a stock saturated CO_2 solution. k_{obs} is independent of variations in $[CO_2]$.

d. $k_{cat} = \frac{k'_{obs} - k_{obs}}{[cat]}$

TABLE 8. Pseudo-First Order Rate Constants for $HCO_3^- + H^+ \rightarrow$ Dehydration[a,b]

pH	k_{obs} (sec^{-1}) [CATALYST] x 10^4 M. [0]	[2.5]	[5.0]	[7.5]	k_{cat} (M^{-1} sec^{-1})[c]
7.40 (HEPES)	0.190±0.001	0.328±0.004	0.426±0.006	0.546±0.009	472±14
7.00 (HEPES)	0.295±0.006	0.419±0.003	0.578±0.004	0.737±0.005	566±20
6.70 (HEPES)	0.455±0.006	0.615±0.012	0.778±0.009	0.918±0.008	646±30
6.40 (HEPES	0.822±0.007	1.019±0.012	1.272±0.025	1.506±0.019	900±64
6.40 (MES)	0.903±0.012	1.103±0.016	1.295±0.021	-	784±66
6.20 (MES)	1.274±0.014	1.434±0.030	1.585±0.022	-	622±72
6.10 (MES)	1.480±0.014	1.581±0.024	1.708±0.035	-	456±98

a. 80%EtOH/H_2O (v/v); 25±0.2°C; $5x10^{-4}$M $NaHCO_3$,$2.5x10^{-2}$M HEPES or MES buffer, I = 0.2M $NaClO_4$.

b. Followed by monitoring the increase in absorbance at 600 nm for BCP indicator ($5x10^{-5}$M), pH determined directly from electrode immersed in solution. pH changes held to 0.04 units.

c. $k_{cat} = \frac{k'_{obs} - k_{obs}}{(cat)}$

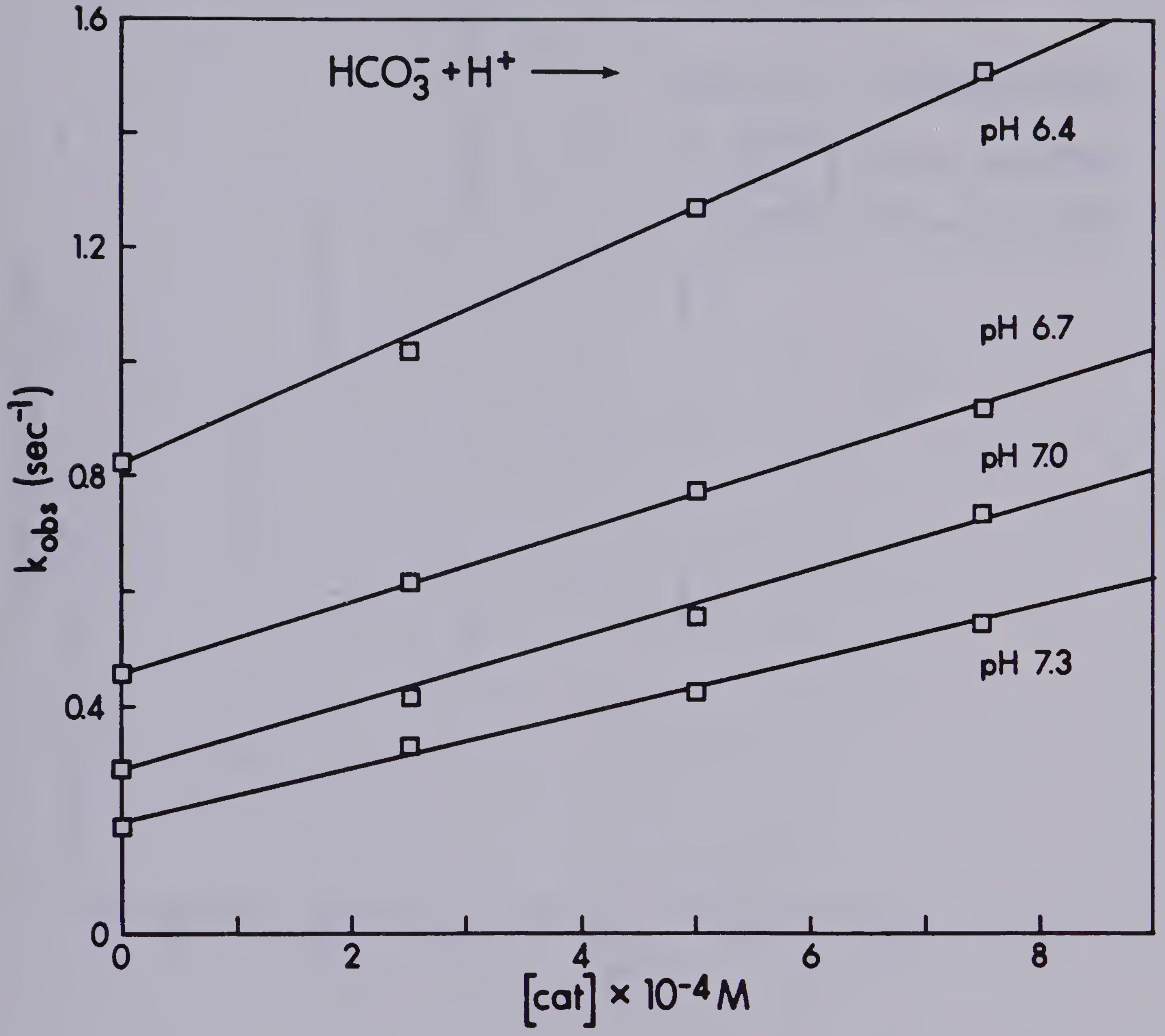


Fig. 8.

Plot of k_{obs} for HCO_3^-/CO_2 equilibration as a function of catalyst concentration and pH, in HEPES buffer.

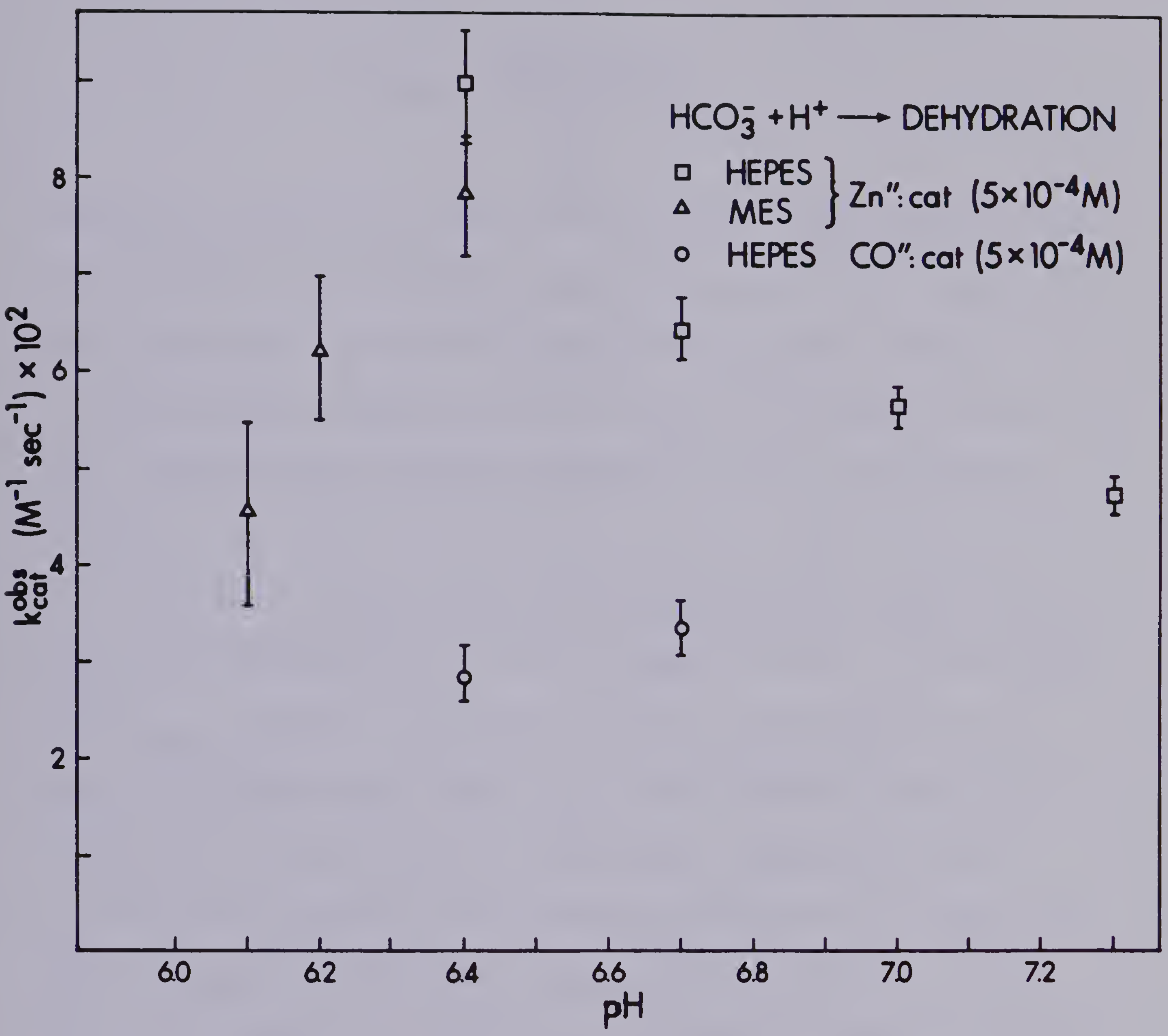


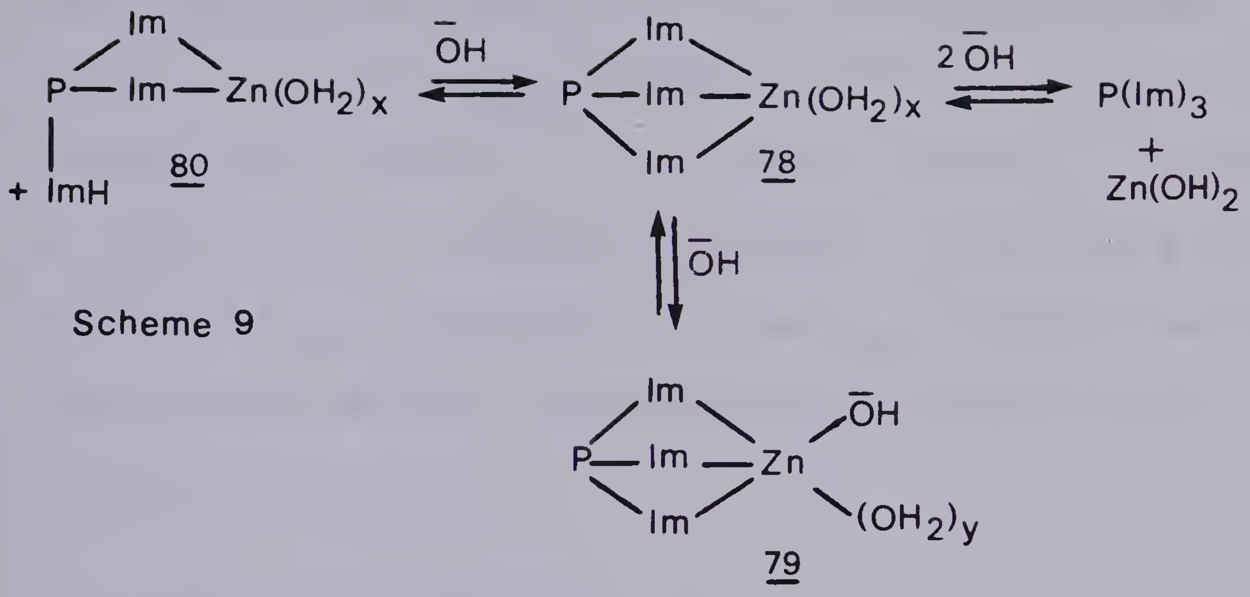
Fig. 9. Second order k_{cat} values for 77:Zn^{++} catalysis of $HCO_3^- \rightleftharpoons CO_2$ under pseudo first order conditions; T = 25 ± 0.2°C; μ = 0.2 M $NaClO_4$, HEPES buffers (□); MES buffers (Δ); 77:Co^{II} (HEPES) (○).

were assessed as a "catalytic rate constant", k_{cat}, which is defined as:

$$k_{cat} = \frac{k'_{obs} - k_{obs}}{[cat]}$$

where k_{obs} and k'_{obs} are the observed rate constants in the absence and presence, respectively of the catalyst. The concentration of the catalytic species, [cat], is determined assuming that all of 77 is bound as the zinc complex (1:1 concentrations of Zn^{++} to 77 being used).

A plot of k_{cat} against pH, approaching equilibrium from the dehydration side, is shown in Fig. 9 and shows that k_{cat} reaches a maximum value at pH 6.40 and reduces at higher and lower pH. This behavior may be a result of a deficiency of the model compound caused by an insufficiently high binding constant of ligand to Zn^{++}. Assuming that the catalytically active species is a 1:1 complex of Zn^{++} to 77, either as the aquo (78) or hydroxide (79) species (or the kinetically equivalent imidazolate aquo species), then at lower pH one imidazole may become protonated and debound as in 80, thus destroying the activity. At higher pH's the precipitation of zinc hydroxide becomes a problem and in fact visible precipitation of what is assumed to be $Zn(OH)_2$ is observed at >7.5 (Scheme 9).



Scheme 9

Thus, one would expect the pH profile depicted in Fig. 9 irrespective of the actual mechanism of catalysis when the binding of Zn^{++} to the ligand is insufficiently large.

Inspection of Tables 7 and 8 shows that in both cases, the observed rate constant, k_{obs}, increases with decreasing pH, as would be expected since the contribution of k_{dehyd} to k_{obs} must be dependent on $[H^+]$ (see eq. 14). However, at any given starting pH, the k_{obs} values obtained starting from opposite sides of the equilibrium are different and always indicate the "CO_2 hydration" reaction to be apparently faster. This may be rationalized by the fact that for HCO_3^- dehydration a proton is consumed, thereby increaseing the pH and so decreasing the rate. In addition, the relative concentrations of the two forms (protonated and

ionized) of the buffer will change during the course of the reaction which will also alter the rate to some extent. Previous workers[126d,127,128] have shown the involvement of buffer in the process, which is supported by the fact that changing the buffer at pH 6.40 gives different k_{obs} (Table 8). A true k_{cat} might be approximated as the average value approaching equilibrium from both directions.

iv. Inhibition studies

It has been demonstrated that the presence of monovalent,[114e] but not divalent,[114e] ions has an inhibitory effect on the action of CA.

The effect of adding halide ions to the zinc complex of 77 at pH 6.40 on the catalytic activity (for "HCO_3 dehydration") was investigated, the observed rate constants being shown in Table 9 and plotted in Fig. 10. For equimolar concentrations of Zn^{++} : 77 : NaX the order of inhibition is $Cl^- > Br^- > I^- > F^-$ ($> ClO_4^-$). For chloride the inhibition is particularly strong and no catalysis at all can be seen when $[cat] = [Cl^-]$. As discussed before, it seems likely that a tightly bound ternary Zn^{++}. 77. Cl^- complex is formed which from this experiment is shown to be inactive. This order of inhibition is different from that found in CA[114e] (I^- $ClO_4^- > Br^- > Cl^- > F^-$) and is probably due (for 77.Zn^{++})

TABLE 9. Inhibitor Effects on k_{obs} for $HCO_3^- + H^+ \rightarrow$ Dehydration[a]

Inhibitor	k_{obs} (sec^{-1}) [Inhibitor]/[Catalyst] 0	 0.5	 1.0	 2.0	 5.0	 10.0
NaCl	1.272±0.025	1.002±0.007	0.820±0.004	-	-	0.818±0.006
NaBr	1.272±0.025	-	0.977±0.006	-	-	-
NaI	1.272±0.025	1.081±0.007	1.018±0.006	0.918±0.016	-	-
NaF	1.272±0.025	-	1.029±0.006	-	-	-
Acetazolamide[b]	1.272±0.025	-	1.234±0.013	1.097±0.007	1.116±0.014	

a. T = 25.0±0.2°C; Ionic strength = 0.2 $NaClO_4$; bromocresol purple indicator, 600 nm, 80% $EtOH/H_2O$; [5×10^{-4} M, Zn:Cat]; maximal pH change < 0.04 units.

b. At high [acetazolamide], kinetic plots differed markedly from first order.

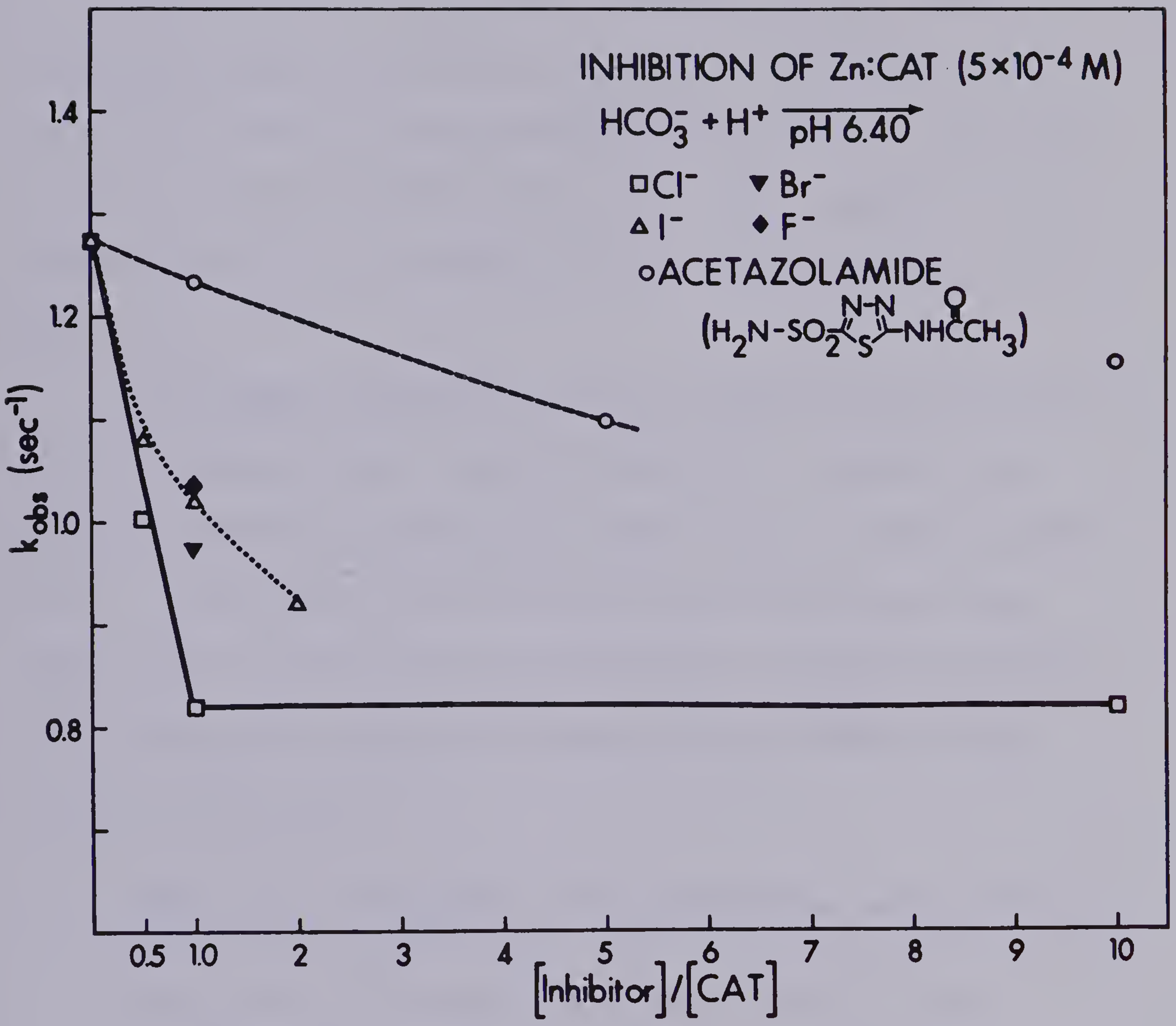


Fig. 10. Effect of inhibitors on k'_{obs} as assessed from HCO_3^- ⟶ dehydration, pH 6.40 HEPES.

to a combination of size and solvation factors. However, no strict comparison can be made between the two sequences since in the latter case the solvent was water.

Br^-, I^- and F^- are less strongly inhibiting allowing rough calculations (based on the residual catalytic activity and assuming that loss of activity is due to the formation of a Zn^{++}.77.X^- complex) for the binding of X^- to the Zn^{++}.77 complex of pK_L's of about 4.

The potent inhibitor of CA, acetazolamide[114a] was found to have little effect (Table 9). Likely its reduced inhibitory effect in this case is due to both fit of the $-SO_2NH_2$ portion into the cavity and the fact that no interaction between the aromatic sulphonamide ring and catalyst (comparable to that in the enzyme) can occur.

v. Catalysis of CO_2/HCO_3^- equilibration with Co^{++}.77

Since the Co^{++} enzyme has about 45% of the activity[129] of the Zn^{++} form, any accurate model might be expected to show both Zn^{++} and Co^{++} activity.

It was found that the Co^{++}.77 complex does show catalytic behavior (Table 10) though the relative effects of zinc and cobalt cannot be assessed since the degrees of binding are somewhat different. The figures quoted in Table 10 for k_{cat} (Co^{++}) are found by assuming that

Table 10 Pseudo First Order Rate Constants for Co.77 catalysed $HCO_3^- + H^+ \longrightarrow$ Dehydration[a] (HEPES buffer)

	k_{obs} (sec^{-1}) (Catalyst) x 10^4 M		k_{cat} ($M^{-1}sec^{-1}$)
pH	(0)	(5)	
6.40	0.822±0.007	0.964±0.005	284±24
6.70	0.455±0.006	0.622±0.007	334±26

Inhibitor Effects of NaCl at pH =6.70

k_{obs} (sec^{-1}) (Cl^-)/(Catalyst) 0	 1	 5	 No Catalyst
0.622±0.007	0.594±0.005	0.542±0.005	0.455±0.006

a. Conditions asiin Table 8.

all the cobalt and ligand are bound strongly but since the binding is rather weak relative to that for zinc, this supposition is tenuous and instead a higher true k_{cat} (Co^{++}) value is to be expected.

The activity of the cobalt complex is inhibited (at pH 6.70) by the presence of NaCl (Table 10). This result is particularly interesting in terms of the fact that whereas the Co^{++} complex of 77 is four co-ordinate in the presence of Cl^- (as evidenced by the visible spectrum) this form has little or no activity in the region studied. However, it has been shown that for the cobalt (II) enzyme a species whose visible spectrum suggests it to be 4 or 5-coordinate[120] appears to be very active in CO_2 hydration. Since the visible spectra of these two systems are different (see Fig. 7) then it is clear that equivalent species are not being compared however, one may note that the presence of four coordinate Co^{++} does not necessarily give rise to a catalytically active species.

Conclusion

The Zn^{++} and Co^{++} complexes of 77 have been shown to be effective in catalyzing the equilibrium between CO_2 and HCO_3^-. It is not possible to compare the relative rates of catalysis of $Zn^{++}.77$ and CA for two reasons. The first is that the k_{cat} values obtained

for the enzyme are for the first order decay of the enzyme-substrate complex whilst in our work a second order constant was determined. Also, whereas in our work the k_{cat} values determined are composite rate constants for attaining equilibrium, including a term for forward and back reactions, two distinct catalytic rate constants are obtained by Michaelis-Menten kinetics i.e. for CO_2 hydration and HCO_3^- dehydration.

Although the metal complexes (Zn^{++}.77 and Co^{++}.77) do not appear to be bound strongly enough to allow a more complete study, e.q. a full pH profile, useful information has been gained from this work.

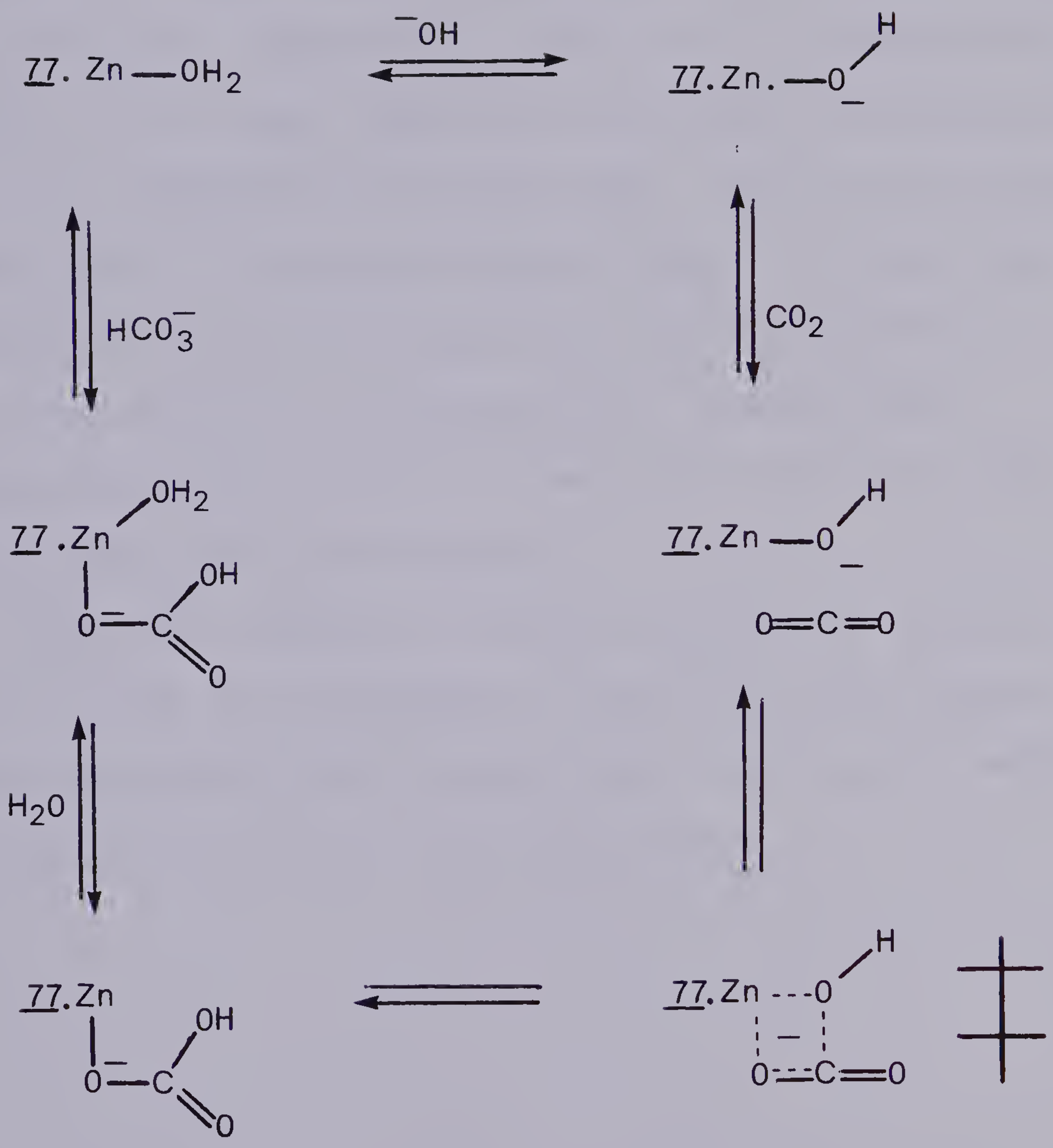
The complex M^{++}.77 is a model for the binding site of the metal in the enzyme and thus any catalysis observed must be due to a chemical reaction of or at this complex. This model does not include any components which may serve to mimic the protein lining to the active site cavity (save perhaps, the high organic content of the medium used). Thus, it can be proposed that the zinc ion can play an active role in the catalysis rather than purely a passive role (vide supra) and that the enzyme catalysis is not purely a result of the enzyme's protein structure (though, of course, this may enhance the effect).

The inhibition studies with NaX are quite informa-

tive. This inhibition can be explained in three ways. One explanation is that the effect is caused by a problem of access to the metal by substrate. The presence of a bound halide hinders the approach of the HCO_3^- ion (or CO_2) which cannot then bind to the metal ion, to form either a four or five coordinate species (this explanation assumes a mechanism which involves a bound substrate). A second explanation, assuming the participation of a bound water molecule, is that an aquo (four or five coordinate) species cannot be formed in this system. A third explanation is that the catalysis involves the ionized form of a coordinated ligand (water or imidazole) and that coordination of anion to the Zn^{++} increases the pK_a of this species, thus raising the effective pH range of the model. Should the last explanation be the true one, the bound water and bound imidazole mechanisms may be assessed using the N-methylated ligand, similar to 77. For this ligand which would contain no N-H groups, the bound imidazolate mechanism is impossible. Unfortunately the N-methylated analogue of 77 itself would not be suitable since the replacement of N-H with $N-CH_3$ gives rise to weaker binding ligands.[102]

A proposed[118e] mechanism of action for Zn^{++}.77 consistent with the above data is presented in Scheme 10. This mechanism is similar to that of Lövgren et al[118e]

Scheme 10


77. Zn—OH2
⁻OH
77.Zn.—O⁻ H
HCO3⁻
CO2
77.Zn OH2 O=C OH O
77.Zn—O⁻ H
O=C=O
H2O
77.Zn O⁻—C OH O
77.Zn---O H O---C=O
‡

except that the H-bonding network between Zn-OH_2 and the protein is absent. The mechanism of hydration of CO_2 is as follows: 1. the bound water ionizes 2. CO_2 diffuses into the inner coordination sheath 3. the hydroxide attacks CO_2 as shown, via a five coordinate zinc intermediate 4. HCO_3^- is freed after subsequent hydrolysis. The reverse reaction, HCO_3^- dehydration is the microscopic reverse and involves: 1. coordination of HCO_3^- to the aquo species followed by elimination of water 2. decomposition of the HCO_3^- ion on the surface of the metal 3. diffusion away of CO_2. It should be noted that one of the features of this mechanism is the occurrence of 5 coordinate Zn^{++} species, this having been demonstrated (at least for Co^{++}) for this system previously (vide supra).

The anion inhibition can be effectively rationalized in terms of replacement of the zinc aquo species by the equivalent halo complex thus reducing the amount of catalytically viable species present.

EXPERIMENTAL

Kinetic experiments were performed on an Aminco-Morrow stopped-flow system, the data being analyzed by an analogue comparison technique.

The solutions were made up as described in the text using a solvent of 80% ethanol, prepared by dilution of 95% ethanol, and used immediately after their preparation. The $NaHCO_3$ solution was made up by dilution of a concentrated stock and the CO_2 solution by dilution of a saturated solution, prepared by bubbling pure CO_2 through 80% ethanol-water containing 0.2 M $NaClO_4$ for 30 minutes. No change in k_{obs} was seen with varying $[CO_2]$ at pH 7.00 over a range 3.33 to 10 fold dilution.

The metal ion solutions were prepared by dilution of concentrated solutions of the perchlorate salt, made by addition of $HClO_4$ to the carbonate salt and standardized by EDTA titration.

The kinetics were monitored at 600 nm, at 25°C (±0.2°) and good first order plots were obtained.

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